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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy.

In trying to understand the mechanism underlying E1A's antitumor activity, we have found that E1A downregulated VEGF expression both in vitro and in vivo. We have also identified additional two new target genes that were critically involved in E1A–mediated chemosensitization.

In addition to the proposed work we have also been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. These studies can provide useful information for us to better understand the molecular functions of E1A, and hopefully we can use this knowledge to better design a mutant E1A construct for cancer gene therapy in the future.

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Table of Contents

Introduction:

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy in the future. The successful reconstruction of this mutant E1A gene will largely depend on our in vitro as well as in vivo studies of the different mutant stable cells.

Body

Statement of Work (no change):

Task 1: To test whether E1A could repress VEGF expression and whether or not mutation of the CR1 domain of E1A will abrogate this activity.

Task 2: To test whther CR1 domain of E1A represses VEGF transcription through recruitment of HDAC-1 via binding with p300

Task 3: To test whether E1A promotes apoptosis through its CR2 domain by disrupting pRB-E2F-1 complexes, releasing free E2F-1.

Task 4: To test the therapeutic effect of the E1A N-terminal mutant as an alternative consctruct for gene therapy in a nude mouse model.

A. Studies and Results:

In the past year we have continued to work on understanding the molecular mechanisms underlying E1A's proapoptotic effect and antitumor activity. Also we have been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. This work may provide useful information for identification of novel target genes of E1A and find new

biomarkers as therapeutic targets. The progress for each aim will be discussed separately as following:

Task 1:

We have observed that stable expression of E1A do repress VEGF expression by Northen blot in cell culture *in vitro* (**Figure 1A**) and by immunohistochemistry in tumor tissue *in vivo* (**Figure 1B**), especially when cells under hypoxia condition (**Figure 1A**). We are currently working on identifying the domains of E1A, which is required for repression of VEGF expression.

Task 2:

We have been working on whether repression of VEGF by expression of E1A is achieved by inhibition of the histone accetyltransferase (HAT) activity of p300 via HDAC1. We have been working on whether expression of E1A could repress the HAT activity of p300. As was originally proposed in the proposal, we have obtained the critical materials for this Task, such as flag-tagged HDAC1 and Gal4-tagged Rb, from Dr. Douglas C. Dean, Washington University School of Medicine, St. Louis, Missouri. Currently, we are working on finding an optimum condition that will help us to detect the E1A-RB-HDAC1-p300 tetraplex or E1A-RB-HDAC1/p300 triplex.

Task3:

In the past year, we have been intensively working on the molecular mechanisms underlying E1A's proapoptotic effect. We have not yet been able to test the role of free E2F-1in E1A–mediated chemosensitization, because some of the antibodies we tried did not work well as we expected. However, in addition to the original proposed experiment we did identify additional two new target genes that were critically involved in E1A–mediated chemosensitization— we found E1A upregulated a proapoptotic factor p38 while downregulating a critical oncogenic survival factor Akt. We demonstrated that activation of p38 and inactivation of Akt were necessary and sufficient for E1A–mediated sensitization to apoptosis induced by serum-starvation, ultraviolet (UV) -irradiation, tumor necrosis factor (TNF) -α, and different categories of anti-cancer drugs, such as adriamycin/doxorubicin, cisplatin, methotrexate, gemcitabine and paclitaxel (Taxol). By screening human cancer cell lines and different types of tumor tissue samples and surrounding normal tissues, we found that the p38 pathway was deregulated in cancer cells due to elevated Akt activation. We also showed that block Akt activation results in elevated p38 phosphorylation and vice versa. We

proposed and tested that a novel feed forward mechanism involving a protein phosphatase PP2A and ASK1, Akt, and p38 kinases regulated Akt and p38 activity, which can be turned on by E1A through upregulation of PP2A activity. A manuscript summarizing the above results was submitted and under communication with Cancer Cell, which is attached for further description and for figures of this work. Also, a patent regarding this novel finding was filed (see appendices).

Recent reports published in Nature Genetics suggested a tumor suppressor role for p38, as inactivation of p38 by a protein phosphatase PPM1D is involved in the development of human cancers by suppressing p53 activation (1,2). PPM1D is also located within a breast cancer amplification epicenter (3), we were interested to see if E1A could affect PPM1D activity. We also wanted to test if Akt will directly affect PPM1D phosphatase activity and indirectly affect p38 activation.

Task 4:

Although we are not at the time to compare the difference of therapeutic efficacy between a mutant E1A construct and the wild–type E1A gene, we have constructed a C-terminal E1A mutant and tested its in vivo effect on tumor growth in animal models. Since we have not been able to establish a stable cell lines with expression of C-terminal E1A, we have constructed a tet-on/off regulated wild–type E1A and C-terminal E1A inducible expression constructs and established tet-off/on inducible cell lines with wild–type E1A or C-terminal E1A expression in breast cancer MCF-7 cells. We are going to study the biological effect of these stable cells and will also compare the therapeutic efficacy of this C-terminal mutant with wild-type E1A and a N-terminal mutant E1A gene in a gene therapy setting.

In addition to the work on the proposed experiment, in corroboration with my colleagues, we uncovered additional two new Akt target genes that associated with drug resistance: the first one is a universal growth inhibitor p21Cip1/WAF1. We found that Akt directly associated with, phosphorylated, and inactivated p21Cip1/WAF1, which correlated with oncogene *Her-2/neu*-mediated drug resistance and cell growth. This work was published in *Nature Cell Biology* (4). In addition, based on our 2-D peptide mapping results and the carboxy-terminal structure of p21Cip1/WAF1, a small peptide was designed, for therapeutic intervention purpose, to compete with endogenous p21Cip1/WAF1 for Akt binding and phosphorylation. Preliminary results showed that it partially reversed sensitivity to drugs of *Her-2/neu* overexpressing cells. Detailed study of this peptide's efficacy and mechanism in drug-sensitization is under way. Meanwhile, our further investigation on Akt activation and drug resistance leads to the discovery of another Akt target gene,

p53, a king tumor suppressor and also a key pro-apoptotic gene, which is inactivated by Akt in breast cancer cells. This finding was also published in *Nature Cell Biology* (5). Further investigations on additional Akt target genes, which may also play a critical role in DNA-damage drug response, are still undergoing.

To gain a global view of genes and proteins interacted with E1A, we also used cDNA microarray and proteomic technologies including 2-D gel electrophoresis and Ciphergen protein chip array to identify the target genes and proteins associated with E1A in breast cancer cells. Differentially expressed genes were found in E1A stable cells versus parental cells by either the cDNA microarray or 2-dimensional gel electrophoresis (**Figure 2A**) and Ciphergen protein chip technology (**Figure 2B**). Further confirmation and characterization of these E1A targeted genes by antibody array and other approaches are underway. Althoguh these experiments are not directly related to the original proposal, they are relevant to E1A's proapoptotic effect and antitumor activity and to breast cancer. Therefore, we will continue to pursue on finding E1A associated molecules and dissect their function on tumor growth and apoptosis in breast cancer.

Key research accomplishments:

(1). We found E1A downregulated VEGF expression in vitro and in vivo.
(2). Evaluated the chemosensitization effect of E1A by systemic E1A gene therapy approach. (See detail in Manuscript).
(3). We have found that upregulation of p38 activity and downregulation of Akt activity are necessary and sufficient for E1A–mediated sentization to apoptosis induced by serum–starvation, TNF-α, UV-irradiation, and different categories of anticancer drugs.
(4). We have found that Akt and p38 can regulate each other.
(5). We identified that a protein phosphatase PP2A participated in E1A–mediated chemosensitization and signal integration between the Akt and p38 pathways.
(6). We have found that deregulation of p38 activity through elevated Akt activation was a general phenomenon in human cancer, including breast and ovarian cancers.

Reportable outcomes:

1. Manuscripts:

   Liao Y, Zou YY, Xia WY, Lee WP, Hung MC: "Reprogramming of deregulated survival and death signals is necessary for E1A–mediated chemosensitization and tumor suppression. " Submitted to Cancer Cell (under communication).

Deng J, Zhang H, Kloosterboer F, **Liao Y,** Klostergaard J, Levitt ML, Hung MC: "Ceramide does not act as a general second messenger for ultraviolet-induced apoptosis." Oncogene. 2002; 21(1): 44-52.

Liao Y*, Zhou BP*, Xia WY, Zou YY, Spohn B, Hung MC: "HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation." *Nature Cell Biol.* 2001; 3(11): 973-82. (*equal contribution)

2. Abstracts and presentations:

Zhou BP, **Liao Y**, Xia WY, Zou YY, Sphon B, and Hung MC: Her-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Proceedings of the Ninety-Second Annual Meeting of the American Association for Cancer Research,* 43: 818, 2002

Liao Y, Zou YY, Xia WY, Hung MC: Chemosensitization by adenovirus E1A through modulation of apoptotic signalings. *Clinical Cancer Research*, 7: 3777s, 2001 and *Proceedings of Molecular Targets and Cancer Therapeutics, AACR-NCI-EORTC Annual Meeting*, Miami Beach, FL, Oct.29-Nov. 2, 2001

Liao Y, Zou YY, Xia WY, Lee WP, Hung MC: "Modulation of apoptotic threshold: enhanced anti-tumor therapy through downregulating Akt and upregulating p38 by adenovirus E1A in human breast cancer cells, " *Proceedings of the Ninety-Second Annual Meeting of the American Association for Cancer Research*, 42: 658-659 (2001).

Liao Y, Spohn B, Lee WP, Zou YY, Hung MC: "Functional domains of adenovirus type 5 E1A in E1A tumor suppression and sensitization to Taxol-induced apoptosis," *Proceedings of the Ninety-first Annual Meeting of the American Association for Cancer Research,* 41:351-352 (2000).

Paul PW, **Liao Y**, Su Z, Spohn B, Ueno N, Lafoe D, Anklesaria P, Hung MC: "In vivo tumor inhibitory activity of the C-terminal fragment of the adenovirus 5 E1A protein," *Proceedings of the 2nd Annual meeting of American Society of Gene Therapy*, P: 176a (1999)

Liao Y, Spohn B., Zou YY, Hung M.C.:"Differential functions of CR1 and CR2 domains of adenovirus type 5 E1A in E1A tumor suppression and sensitization to Taxol-inducced apoptosis." Proceedings of the *42nd Annual Clinical Conference/52nd Annual Symposium on Fundamental Cancer Research,* P: 138-139 (2000)

Liao Y, Spohn B., Li WP, Zou YY, Hung M.C: "Differential functions of CR1 and CR2 domains of adenovirus type 5 E1A in E1A tumor suppression and sensitization to Taxol-inducced apoptosis." *Proceedings of the University of Texas M.D. Anderson Cancer Center Trainee Recognition Day and Research Exposition*, P: B33 (2000)

3. Patent (pending):

Mien-Chie Hung and **Yong Liao**: Compositions and methods for inactivating the Akt oncogene and activating the p38 pro-apoptotic gene. (MDA00-050)

Applied for coverage under U.S. Patent (Serial No: 60/277,788 and Application No. 10/103.542), Chinese Patent (No. 011117869), and Taiwanese Counterpart Patent (N0. 90106723)

Conclusion:

These studies on the molecular mechanisms underlying E1A's tumor suppression and chemosensitization can help us to better design alternative E1A constructs for cancer gene therapy. Specifically, identification of additional E1A target genes, such as PP2A, Akt and p38, may help us in finding novel ways to treat cancer patients by targeting the deregulated signals. The combination of E1A gene therapy with Taxol or other chemotherapeutic drugs is one potential new therapeutic approach for the treatment of cancer patients, as we have shown in the animal models. The mutant E1A construct, if it works in animal model, could potentially be translated into the clinic and be of great benefit to breast cancer patients.

Reference:

1. Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ, Appella E. Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet. 2002 Jun;31(2):210-5.

2. Li J, Yang Y, Peng Y, Austin RJ, Van Eyndhoven WG, Nguyen KC, Gabriele T, McCurrach ME, Marks JR, Hoey T, Lowe SW, Powers S. Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. Nat Genet. 2002 Jun;31(2):133-4.

3. Choi J, Appella E, Donehower LA. The structure and expression of the murine wildtype p53-induced phosphatase 1 (Wip1) gene. Genomics. 2000 Mar 15;64(3):298-306.

4. Zhou BP, Liao Y, Xia WY, Zou YY, Spohn B, Hung MC: "HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation." *Nature Cell Biol.* 2001; 3(11): 973-82.

5. Zhou BP, Liao Y, Xia WY, Spohn B, Lee MH, Hung MC: "Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells." *Nature Cell Biology* 2001; 3(3): 245-252

Figure 1. E1A downregulate VEGF expression *in vitro* (A) and *in vivo (B).*

A. Northern Blot analysis of VEGF expression in cell culture *in vitro*

B. Immunohistochemistry analysis of VEGF Expression in tumor tissue *in vivo*

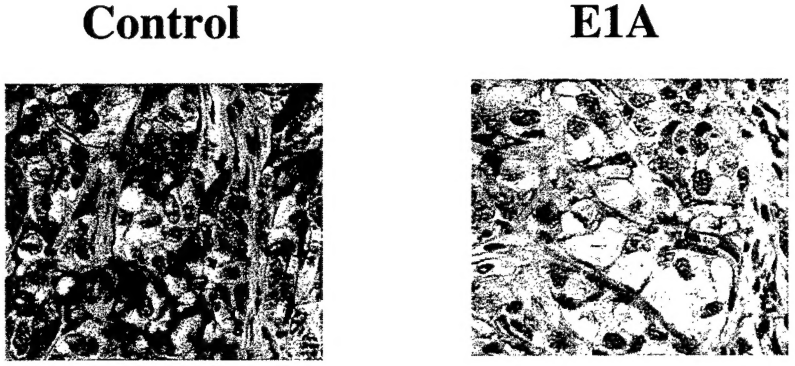
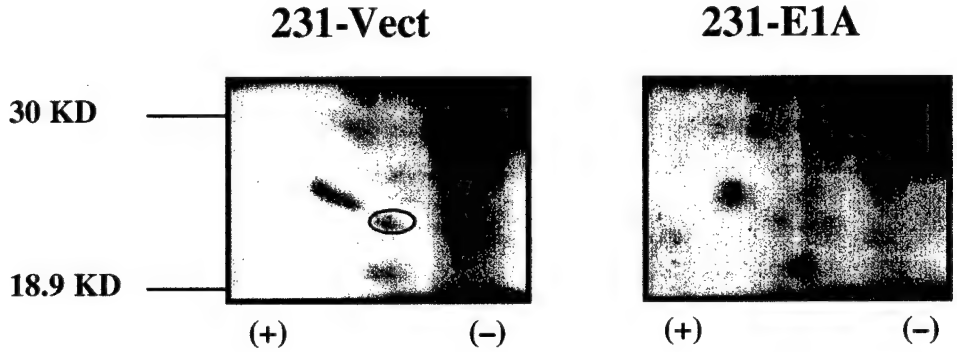


Figure 2. Proteins differentially expressed in 231-E1A stable cells identified using 2D gel (A) and Ciphergen protein profiling analysis (B).

A. E1A down-regulated proteins identified by 2D gel electropheresis.



B. Differentially expressed proteins in 231-E1A cells identified by Ciphergen proteinchip analysis

Type of Protein chip	Up-regulated proteins (KD)	Down-regulated proteins (KD)
H4	37.5, 43.7, 48.8, 53.1, 87.4, 4.36, 6.81	45.4, 56.5, 65.7, 69.7, 6.25
SAX2	34.9, 61.1, 7.9	12.4, 17.5, 25.9, 148.8, 6.18
WCX2	37.4, 3.3	69.7
IMAC-cu	37.3, 40.6	49.6, 65.7

Reprogramming of deregulated survival and death signals is necessary for E1A-mediated chemosensitization and tumor suppression

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Summary

Most human cancer cells are associated with activation of the critical oncogenic survival factor Akt. We demonstrated that activation of p38 and inactivation of Akt were necessary and sufficient for the adenoviral early region 1A (E1A)-mediated drug sensitization. By screening human cancer cell lines and different types of tumor tissue samples and surrounding normal tissues, we found that the p38 pathway was deregulated in cancer cells, partly due to elevated Akt activation. We also showed that block Akt activation results in elevated p38 phosphorylation and vice versa. We proposed and tested that a feed forward mechanism involving a phosphatase PP2A and ASK1, Akt, and p38 kinases regulated Akt and p38 activity, which can be turned on by E1A through upregulation of PP2A activity. Thus, reprogramming of the deregulated survival and death signals is necessary for tumor suppression and drug sensitization by E1A.

Significance

Deregulation of the survival and death signals contributes to the altered response of cancer cellls to drugs. The adenoviral E1A protein, which is currently being tested as an anti-tumor gene in multiple clinical trials, reprogrammed the deregulated signals through activation of the phosphatase PP2A, which resulted in reactivation of the proapoptotic factor p38 through repression of the oncogenic survival factor Akt via a feed-forward mechanism. In addition, we first showed that inactivation of p38 resulting from elevated Akt activation was a general phenomenon found in most of the human cancer cell lines and tissues we tested. Thus, reprogramming the deregulated survival and death signals is necessary for drug sensitization and tumor suppression. The identified mechanism may be important for the development of therapeutic strategies by targeting the deregulated signals for cancer treatment.

Introduction

Cancers are developed from deregulated cell growth. This deregulation occurs as a result of the imbalance between cell proliferation and cell death, or apoptosis. Many oncogenes and tumor suppressors mediate their effects by interfering with or inducing apoptotic signaling; thus, apoptotic pathways may be significantly altered in cancer cells relative to untransformed cells (Evan and Vousden, 2001; Green and Evan, 2002; Houghton, 1999; Johnstone et al., 2002; Reed, 1999; Stambolic et al., 1999; Tsatsanis and Spandidos, 2000). For example, it has been shown that the serine/threonine kinase Akt and its family members Akt 2, and 3 are amplified or their activity is constitutively elevated in human carcinomas such as breast, pancreatic, ovarian, brain, prostate, and gastric adenocarcinomas (Bellacosa et al., 1995; Kaname and Roth, 1999; Kandel and Hay, 1999; Nicholson and Anderson, 2002; Testa and Bellacosa, 2001). As it is a direct downstream target of phosphoinositol 3-kinase (PI3K), Akt is also a key oncogenic survival factor and can phosphorylate and inactivate a panel of critical proapoptotic molecules, including Bad, caspase-9, the Forkhead transcription factor FKHRL1, GSK3-β, etc. (Kandel and Hay, 1999; Nicholson and Anderson, 2002; Scheid and Woodgett, 2001; Testa and Bellacosa, 2001). Also, activation of Akt has been shown to induce resistance to apoptosis induced by a range of drugs (Page et al., 2000). Thus, molecules that can block Akt activity may have important significance in cancer therapy and drug sensitization (Harbour, 2000; Huang, 2001; Yu and Hung, 2000).

In addition to the PI3K-Akt oncogenic survival pathway, two other intrinsic

signaling pathways are also involved in regulating cellular response to apoptosis: the Jun N-terminal kinase (JNK) pathway and the p38 MAP kinase proapoptotic pathway (Davis, 2000; Ichijo, 1999; Stambolic et al., 1999). Whether a cell lives or dies in response to an apoptotic stimulus is largely dependent on the balance of intrinsic survival signals and proapoptotic signals within it (Bamford et al., 2000; Nicholson, 2000; Schmitt, 1999). How the proapoptotic and antiapoptotic signals are integrated within a cell in response to an apoptotic stimulus is not clear.

The adenovirus early region 1A (E1A) proteins were originally described as immortalizing oncoproteins that could transform rodent cells in cooperation with other oncogenes, however, we and other groups have subsequently shown that the adenovirus type 5 E1A proteins have antitumor activities in different types of human cancers. The tumor suppression apparently results from the ability of E1A to re-program transcription in tumor cells, although the molecular basis of this effect remains unclear (Frisch, 2001b; Frisch and Mymryk, 2002). In addition, E1A has been shown to induce chemosensitization to different categories of anti-cancer drugs, including cisplatin, adriamycin, etoposide, staurosporine, 5-fluorouracil, and paclitaxel (Taxol) (Brader et al., 1997; Duelli, 2000; Frisch, 1995; Lowe et al., 1993; Sanchez-Prieto et al., 1995; Ueno et al., 1997). Because the primary targets of these drugs are different, there may be a general cellular mechanism that mediates chemosensitization that can be activated by E1A. The E1A gene has been introduced into cancer patients in a few clinical trials to test its antitumor activity (Anklesaria, 2000; Benjamin et al., 2001; Hortobagyi et al., 2001; Hung et al., 2000; Khuri et al., 2000; Yoo et al., 2001), and encouraging therapeutic results were recently reported after using a combination of chemotherapy and the ONYX-

015 virus, in which the wild-type E1A gene is retained (Khuri et al., 2000). However, the molecular mechanisms underlying E1A-mediated sensitization to apoptosis have not been completely defined.

In this study, we found that E1A-mediated sensitization to apoptosis is achieved by upregulating p38 activity through repression of Akt activity. We demonstrate herein that regulation of Akt and p38 pathways is a physiological phenomenon and is mediated by a feed-forward mechanism involving the protein phosphatase PP2A. In Akt knock out MEF cells, the level of p38 phosphorylation was increased, while it was undetectable in constitutively active myr-Akt transfected stable cells. In addition, inactivation of p38 through the elevation of Akt activation was also detected in different types of human cancer cell lines and tumor tissues, indicating that deregulation of the Akt and p38 pathways is a common phenomenon in human cancer and may contribute to drug resistance in cancer cell. Thus, reprogramming of a major deregulated cellular signal pathway by E1A is necessary for its antitumor activity and drug sensitization.

Results

E1A sensitizes cells to Taxol-induced apoptosis in vitro

Taxol is a promising frontline chemotherapeutic agent for treating breast and ovarian cancers. To test whether E1A can sensitize cells to Taxol-induced apoptosis, we treated MDA-MB-231 and MCF-7 human breast cancer cell lines and their E1A-expressing cells with Taxol and performed MTT cytotoxicity assays. We found that stable expression of wild-type E1A enhanced sensitivity to Taxol-induced killing in MDA-MB-231 cells by more than 10-fold when compared with Taxol-treated parental or vector-transfected

control cells (Figure 1A). Stable expression of E1A also enhanced the sensitivity of MCF-7 cells to Taxol, although to a lesser extent. Interestingly, a revertant of the MCF-7 clone, that lost its E1A expression during cell culture also lost its sensitivity to Taxol (Figure 1B). Three additional independent E1A-stable clones from both cell lines also showed similar sensitivity to Taxol (data not shown).

To address whether apoptosis was involved in E1A-mediated sensitization to Taxol, we examined the presence of poly (ADP-ribose) polymerase (PARP) cleavage as an apoptotic cell death marker. We detected a dose-dependent increase of PARP cleavage in E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells upon treatment with 0.1 μM to 0.001 μM Taxol. In parental and 231-Vect cells, however, PARP cleavage was observed only upon treatment with 0.1 μM Taxol, while in the revertant MCF-7 cell clone, it was not detected at all (Figures 1A and B). Fluorescence-activated cell sorting (FACS) analysis also supported that E1A enhanced Taxol-induced apoptosis, as demonstrated by an increased proportion of sub-G1-phase cells in E1A-expressing cell lines (Figure 1C). Taxol is well known to induce G2/M arrest (as we observed in parental and 231-Vect cells), but we noticed that expression of E1A altered Taxol-induced G2/M arrest, suggesting that E1A may affect the cell cycle distribution through a mechanism yet to be determined. Nevertheless, the results described above clearly indicate that E1A sensitized Taxol-induced apoptosis in vitro.

E1A gene therapy sensitizes cells to a Taxol-induced antitumor effect in vivo

To test whether E1A also mediated sensitization to Taxol in vivo, we used a deoxynucleotide transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay to

compare Taxol-induced apoptosis in tumor tissue samples obtained from mice inoculated with 231-E1A and 231-Vect cells. We found that Taxol-treated 231-E1A tumor tissues had a significantly higher percentage of TUNEL-positive cells than Taxol–treated 231-Vect tumor tissues and control normal saline-treated tumors did (Figure 2A and B). We then evaluated tumor size. In the absence of Taxol treatment, the mean tumor volume in the E1A–inoculated animals was significantly lower than that in the control 231-Vect–inoculated animals ($P < 0.01$). However, Taxol treatment produced dramatic regression of 231-E1A tumors; the mean 231-E1A tumor volume in the Taxol-treated group was just 21.7% of that in the control saline-treated 231-E1A group (Figure 2C), while the mean tumor volume in 231-Vect–inoculated animals still reached ~50% of that in the control group. When comparing tumors in Taxol–treated 231-Vect–inoculated animals with those in the Taxol–treated 231-E1A group, the latter were more sensitive to Taxol (21.7% vs. 50% reduction; $P < 0.01$). These results indicate that E1A sensitized cells to Taxol-induced apoptosis in vivo and inhibited mammary tumor development in the animals.

To explore whether E1A directly enhances Taxol-induced apoptosis in a gene therapy setting, we used a systemic SN-liposome (Zou et al., 2002) to deliver the E1A gene by *i.v.* injection via the tail vein in an established orthotopic mammary tumor model of animals inoculated with MDA-MB-231 cells. Compared with treatment using an E1A-liposome or Taxol alone, a combination of E1A gene therapy and Taxol chemotherapy significantly enhanced the therapeutic efficacy and dramatically repressed tumor growth ($P < 0.01$) (Figure 2D). The tumors were completely eradicated in three of seven mice treated using combination therapy. In addition, survival rates were significantly higher in

animals treated with combination therapy than in those treated using the SN-liposome, E1A-liposome, or Taxol alone (Figure 2E); three of the seven animals that received combination therapy had a tumor-free survival duration of more that one year. The data clearly show that expression of E1A significantly enhanced Taxol's antitumor effect and prolonged survival rates in this orthotopic animal model.

E1A upregulates p38 activity and downregulates Akt activity

To determine whether apoptosis-related signaling molecules are involved in E1A-mediated sensitization to Taxol, we examined the phosphorylation status of the three well-known kinases involved in regulation of apoptosis – p38, Akt, and JNK – in 231-E1A and MCF-7-E1A cells versus vector-transfected cells. We detected phosphorylated p38 in cells stably expressing E1A but not in vector-transfected cells. However, the level of phosphorylated Akt was much higher in vector-transfected cells than in E1A-expressing cells. The levels of total Akt and p38 were similar in both types of cells (Figure 3A). Kinase assays showed that the p38 activity was higher and the Akt activity was lower in 231-E1A cells than in 231-Vect cells (Figures 3B and 3C). We did not detect any differences in the level of phosphorylated JNK between E1A-expressing and vector-transfected cells (data not shown). These results indicate that E1A enhanced the activity of the proapoptotic kinase p38 and repressed the activity of the antiapoptotic kinase Akt, but did not affect JNK phosphorylation.

Activating p38 and inactivating Akt are required for E1A-mediated sensitization to Taxol-induced apoptosis

To evaluate whether activation of p38 is required for E1A-mediated sensitization to

Taxol, we tested whether blocking p38 activity will block E1A-mediated sensitization in 231-E1A cells. We used the specific p38 inhibitor SB203580 (Figure 4A) and a dominant-negative p38 (DN-p38) mutant to block p38 activation (Figure 4B). We found that SB203580 on concentration of 20 μM was sufficient to block p38 activation in 231-E1A cells. In the absence of SB203580, the level of phosphorylated p38 increased after exposure to Taxol (Figure 4C, lanes 1–2). In contrast, pretreatment using SB203580 inhibited the phosphorylation of p38 in cells with or without exposure to Taxol (Figure 4C, lanes 3–4). We also measured luciferase activity as an index of cell survival by transfection of the pcDNA3-Luc reporter construct into 231-E1A cells. In the absence of SB203580, the luciferase activity, which represented cell viability, in Taxol-treated cells decreased to 38% of that in untreated control cells; when cells were pretreated with SB203580, Taxol inhibited luciferase activity to a lesser extent (Figure 4C, lanes 1–4). In addition, FACS analysis showed that pretreatment using SB203580 protected 231-E1A cells from Taxol-induced apoptosis (27.5% vs. 18.0%) (Figure 4D, lanes 2 and 4). These data suggest that p38 activation was required for E1A-mediated sensitization to Taxol-induced apoptosis.

To further support the requirement of p38 activation for E1A-mediated sensitization to Taxol-induced apoptosis, we next examined whether suppression of p38 activity by the DN–p38 mutant could also block Taxol-induced apoptosis in 231-E1A cells. To that end, isopropyl-β-D-thiogalacto-pyranoside (IPTG)-inducible, Flag-tagged DN-p38 stable cell clones were established in 231-E1A cells. When the cells were switched from their normal medium to medium containing 5 μM IPTG for 24 hr, expression of Flag-tagged DN-p38 was induced, but the level of endogenous p38 was not changed (Figure 4B, right

panel). Also, FACS analysis showed that induction of DN-p38 by IPTG significantly inhibited Taxol-induced apoptosis (Figure 4B, upper panel). Identical results were obtained when two additional stable clones were studied (data not shown). However, IPTG could not induce this effect in the 231-E1A cells without DN-p38 (Figure 4B, left panel). These results further support that upregulation of p38 activity was required for E1A-mediated sensitization to Taxol and that blocking of p38 activity inhibited this sensitization.

To determine whether downregulation of Akt activity is also required for E1A-mediated sensitization to Taxol, we examined whether activation of Akt would inhibit Taxol-induced apoptosis in 231-E1A cells. To that end, a hemagglutinin A (HA)-tagged, myristoylated, membrane-targeted constitutively active Akt construct (CA-Akt) and pcDNA3-luciferase reporter construct (pcDNA3-Luc) were cotransfected into 231-E1A cells. Expression of CA-Akt was detected using an anti-HA monoclonal antibody (Fig. 4C, lanes 5 and 6). The level of phosphorylated Akt was increased in CA-Akt–transfected 231-E1A cells compared with that in control 231-E1A cells (Figure 4C, lanse 1and 2 vs. 5 and 6). Also, luciferase activity was significantly higher in cells transfected with CA-Akt than in control 231-E1A cells after exposure to Taxol (Figure 4C, lane 2 vs. lane 6). Also, FACS analysis showed that fewer apoptotic cells were detected in CA-Akt–transfected cells (15.9%) than in control 231-E1A cells (27.5%) after exposure to Taxol (Figure 4D, lane 2 vs. lane 6). Thus, inhibition of Akt phosphorylation was also found to be required for E1A-mediated sensitization to Taxol-induced apoptosis.

Increasing p38 activity or blocking Akt activity enhances Taxol-induced apoptosis

To test whether increasing p38 activity is sufficient to enhance Taxol-induced apoptosis, we enhanced p38 activity by transfectiing an HA-tagged p38 construct (HA-p38) along with the reporter construct pcDNA3-Luc into MDA-MB-231 cells and analyzed whether increased p38 activity sensitizes Taxol-induced apoptosis. After transfection, cells were grown for 36 hr and then exposed to Taxol. Compared with cells transfected with pcDNA3-Luc alone, those cotransfected with HA-p38 showed an increased level of phosphorylated p38 and decreased luciferase activity upon exposure to Taxol (Figure 5A, lanes 1 and 2 vs. 3 and 4). FACS analysis also showed that the percentage of apoptotic HA-p38–transfected cells increased to 14.9% after exposure to Taxol (Figure 5A, bottom panel). These results indicate that increasing p38 activity was sufficient to enhance the sensitivity of MDA-MB-231 cells to Taxol-induced apoptosis.

In addition, to test whether blocking of Akt activity enhanced the sensitivity of cells to Taxol, both biochemical and genetic approaches were employed. First, we demonstrated that wortmannin, a specific inhibitor of the PI3K pathway, blocked Akt phosphorylation in a dose–dependent manner (Figure 5B). We transfected pcDNA3-Luc into MDA-MB-231cells and used luciferase activity as a measurement of cell survival. In the presence of wortmannin, Akt phosphorylation was blocked, (Figure 5C, lanes 1and 2 vs. 5 and 6) and luciferase activity decreased by almost 50% after exposure to Taxol, compared with that in cells treated using the same dose of Taxol but in the absence of wortmannin (Figure 5C, lanes 1 and 2 vs. 5 and 6). FACS analysis showed that a higher percentage of wortmannin-treated cells than control cells (11.9% vs. 1.4%) underwent apoptosis in response to Taxol treatment (Figure 5D, lane 2 vs 6). Alternatively, when a dominant-negative Akt construct (DN-Akt) and pcDNA3-Luc were transiently

cotransfected into MDA-MB-231 cells, similar results were obtained; specifically, the level of phosphorylated Akt decreased (Figure 5C, lanes 1and 2 vs. 3 and 4). In the presence of DN-Akt, less viable cells were detected by luciferase assay after exposure to Taxol (Figure 5C, lanes 2 and 4). FACS analysis also showed that after exposure to Taxol, the percentage of DN-Akt–transfected cells that underwent apoptosis increased from 1.4% to 8.4% (Figure 5D, lanes 2 vs. 4). Thus, blocking of Akt activity enhanced sensitivity to Taxol-induced apoptosis.

The same mechanism for E1A-mediated sensitization to drug-induced apoptosis applies to apoptosis induced by serum starvation, TNF-α, and UV-irradiation

To determine whether the same mechanism of E1A-mediated sensitization to Taxol applies to other anticancer drugs, we tested the effects of four additional drugs used in the clinic for treatment of human cancer thought to induce antitumor activities through different modes of action: doxorubicin/adriamycin (topoisomerase II inhibitor), cisplatin (DNA-damaging agent), methotrexate (antimetabolite drug), and gemcitabine (antimetabolite drug). Expression of E1A significantly enhanced each drug's cytotoxicity in MDA-MB-231 cells as determined using the MTT assay (Figure 6A). In addition, PARP cleavage was observed in 231-E1A cells but not in 231-Vect cells treated using each of the drugs. Downregulation of Akt phosphorylation and upregulation of p38 phosphorylation were also detected in E1A-expressing cells after 24 hr of exposure to each drug (Figure 6B). These results suggest that upregulation of p38 phosphorylation and downregulation of Akt phosphorylation may contribute to E1A-mediated sensitization to apoptosis induced by these drugs. It is worth mentioning that the level of

phosphorylated Akt and p38 were also affected in the drug-treated control cells not expressing of E1A (e.g., gemcitabine and adriamycin) (Figure 6B, lanes 5 and 9).

To address whether upregulation of p38 phosphorylation and downregulation of Akt phosphorylation also applied to drug-induced apoptosis in the absence of E1A, we tested whether Taxol alone could enhance p38 phosphorylation and inhibit Akt phosphorylation, which would then contribute to Taxol-induced apoptosis. Although Taxol at the lower concentration (0.001 μM) did not induce apoptosis or change the level of phosphorylated p38 or Akt (Figures 5A, 5C, and 5D, lane 2), we found that it was able to induce apoptosis as well as enhance p38 phosphorylation and inhibite Akt phosphorylation at the higher concentration (0.1 μM) (Figure 6C, left panel). When MDA-MB-231 and HBL-100 cells were exposed to higher dose of gemcitabine (20 μg/ml) or higher concentration of adriamycin (20 μM), we observed a similar pattern of downregulation of Akt and upregulation of p38 phosphorylation, which are correlated with PARP cleavage (Figure 6C, right panel). The results suggest that upregulating p38 phosphorylation and downregulating Akt phosphorylation may not be limited to E1A-mediated sensitization to apoptosis, but may also contribute to drug-induced apoptosis in the absence of E1A. Thus, downregulating Akt activity and upregulating p38 activity may represent a general cellular mechanism of response to chemotherapeutic drug-induced apoptosis, and E1A may turn on this cellular mechanism and mediate sensitization to apoptosis induced by different chemotherapeutic drugs.

To determine the physiological relevance of downregulation of Akt phosphorylation and upregulation of p38 phosphorylation in the execution of apoptosis, we extended our investigation to apoptosis induced by serum starvation, TNF-α, and UV-irradiation. We

observed that downregulation of Akt phosphorylation and upregulation of p38 phosphorylation were correlated with serum starvation–, TNF-α–, and UV–induced PARP cleavage in MDA-MB-231 or 231-E1A cells, especially detached apoptotic cells. However, a dose of 10 times greater than that in 231-E1A cells are required for inducing a similar response in parental cells (Figure 6D, right panel). Taken together, downregulation of Akt phosphorylation and upregulation of p38 phosphorylation may represent a general mechanism apoptosis in responding to different apoptotic stimuli, including serum starvation, UV-irradiation, TNF-α, and different categories of anticancer drugs.

Physiological regulation of the Akt and p38 pathways and the deregulation in human cancers

To investigate the relationship between the p38 and Akt signaling pathways, we first sought to determine whether p38 could be upstream from Akt. We used SB203580 to block p38 activation and examined its effect on Akt phosphorylation in 231-E1A cells. When the level of phosphorylated p38 decreased, the level of phosphorylated Akt increased at 8 hr. Once the level of phosphorylated p38 recovered at both 16 and 24 hr, the level of phosphorylated Akt decreased again (Figure 7A). These results indicate that p38 phosphorylation was required for repressing Akt phosphorylation, suggesting that the former was upstream from the later. This conclusion was supported by the results using DN-p38 transfectants (Figure 4B). Once DN-p38 was induced to block p38 kinase activity by IPTG, the level of phosphorylated Akt remained high, but in the absence of IPTG, the level of phosphorylated Akt was reduced. This effect was even more dramatic

when cells were treated using Taxol. Additionally, to determine whether Akt activation could also affect p38 phosphorylation, we used wortmannin to block Akt activation and examined its effect on p38 phosphorylation in MDA-MB-231 cells. Interestingly, we observed that blockage of Akt phosphorylation enhanced p38 phosphorylation (Figure 7B), suggesting that Akt could also be upstream of p38. The results described above suggest that inactivation of the proapoptotic factor p38 by its dephosphorylation may activate anti-apoptotic factor Akt by its phosphorylation. Inactivation of Akt activity may also activate p38 activity through regulation of phosphorylation. Thus, Akt and p38 can regulate each other.

The experiments described above established the potential for a negative feedback regulation of the Akt and p38 pathways. To test whether this regulation has physiological significance, we measured the level of phosphorylated Akt and p38 in Akt1 knockout mouse embryo fibroblast (MEF) cells and myr-Akt1 transfected stable cells. As expected, we observed that the level of phosphorylated p38 was increased in Akt1 knockout MEF cells compared with that in Akt (+/+) and Akt (+/–) MEF cells. Furthermore, the phospho-p38 protein was undetectable in the myr-Akt1 stable cells regardless of the high level of total p38 protein (Figure 7C). These results are consistent with the data obtained using a chemical inhibitor to block Akt activation (Figure 4B and 4C, Figure 7A and 7B) and further suggest that the negative feedback regulation of the Akt and p38 pathways is a general physiological phenomenon.

To explore whether this negative feedback mechanism has a pathophysiological role in human cancer, we analyzed the activity of Akt and p38 in naturally occurring human cancer cells by screening a panel of breast, ovarian, prostate, pancreatic, and colorectal

cancer cell lines. We found that Akt was constitutively activated, while p38 activity was undetectable in most of these cancer cell lines (Figure 7D). Using tissue array slides, we obtained similar results by screening normal organ tissues and tumor tissues of different origins (Figure 7E). We found that the phospho-Akt level was dramatically higher, while phospho-p38 was undetectable in most of the cancer tissues which were obtained from different types of solid tumors, such as cancers from breast, lung, liver, bile duct, gastric, colorectal, renal cell, ovarian, and uterine cancer; and, malignant lymphoma; and Schwannoma. In contrast, the intensity of phospho-p38 protein staining was relatively strong, while that of phospho-Akt staining was very weak in normal organs and parallel normal tissues. Representative data on the expression of phospho-p38 and phospho-Akt in normal versus tumor tissues obtained from the breast, lung, liver, and biliary duct are shown in Figure 7E. In addition, we noticed that unlike the phospho-p38 staining in normal tissues, which was predominantly present in the nucleus or nucleus and cytoplasm compartments, phospho-p38 staining in tumor tissues, if positive, was usually weak and predominantly present in the cytoplasm. Further analysis of 50 cases breast cancer at different stages— 4 at stage I (T1), 21 at stage II (T2), 20 at stage III (T3) and 5 at stage IV (T4) — showed that the relative intensity of active Akt staining was closely correlated with the tumor stage, while the relative intensity of phospho-p38 staining was inversely correlated with the tumor stage (Figure 7F). In the T3 and T4 cases, 80% of the samples were negative for phospho-p38 staining, while 100% were positive for phospho-Akt staining (Figure 7G). Because phosphorylated p38 could be detected in MEF cells and most of the normal organ tissues but not in most of the cancer tissues or cell lines, the Akt and p38 pathways may be deregulated in these human cancer cells, and inactivation

of p38 may result from the elevated Akt activation.

E1A enhances PP2A activity, which regulates the Akt/ASK1/p38 kinase cascade via dephosphorylation of Akt

In an attempt to understand how p38 and Akt regulate each other, we sought to determine whether Akt is physically associated with p38 using coimmunoprecipitation experiments. Specifically, CA-Akt was transiently transfected into both MDA-MB-231 and 293T cells. The cells were lysed for about 30 hr after transfection, and Akt and/or p38 was immunoprecipitated. We did not detect p38 in immunoprecipitated Akt samples (Figure 8A) or Akt in immunoprecipitated p38 samples (data not shown), suggesting that Akt and p38 were not directly associated under the conditions we used. Because ASK1 is an upstream kinase known to phosphorylate p38 (Ichijo et al., 1997; Tobiume et al., 2001) and there is a consensus Akt phosphorylation site in the ASK1 protein sequence, we next tested whether ASK1 could bind to and be inactivated by Akt. The results showed that ASK1 was coimmunoprecipitated with Akt (Figure 8A). While this experiment is ongoing, a recent report indicated that ASK1 is indeed a substrate of Akt (Kim et al., 2001). Together with the fact that ASK1 has been shown to directly phosphorylate p38 (Ichijo et al., 1997; Tobiume et al., 2001), Akt, ASK1, and p38 may form a kinase cascade to regulate apoptosis.

We next asked by which mechanism E1A affects Akt phosphorylation. Because phosphorylation of protein kinases is tightly regulated by related protein phosphatases (Barford et al., 1998; Keyse, 2000), we measured PP2A and PP2C serine/threonine phosphatase activity in stable E1A-expressing cells versus that in 231-Vect cells using

protein phosphatase assays. We did not measure the activity of PP2B, as it is calcium-dependent and irrelevant to the current study (Barford et al., 1998; Keyse, 2000; Zolnierowicz, 2000). We observed that E1A expression enhanced PP2A activity (Figure 8B) but no significant difference in PP2C activity (data not shown). We then tested E1A to see whether it could increase the level of expression of the members of the PP2A phosphatase complex. Although the level of expression of the regulatory subunit of PP2A, PP2A/A, was not significantly different in E1A-expressing and 231-Vect cells, we did detect notably higher expression of its catalytic subunit, PP2A/C, in E1A-expressing cells in the presence or absence of Taxol (Figure 8C). Also, to determine whether PP2A affects Akt phosphorylation, we used the specific PP2A inhibitor okadaic acid to block PP2A activity and measured Akt phosphorylation in E1A-expressing cells. We found that blocking PP2A activity clearly enhanced Akt phosphorylation (Figure 8D). Consistent with the results shown in Figure 7A, p38 phosphorylation was inversely correlated with Akt phosphorylation (Figure 8D). To verify whether PP2A could directly dephosphorylate Akt, we measured the PP2A phosphatase activity using Akt as a substrate. Endogenous Akt was immunoprecipitated and incubated with purified human PP2A enzyme (hPP2A). As measured by anti-phospho-Akt antibody, dephosphorylation of Akt occurred in a PP2A dose-dependent manner and could be completely blocked by okadaic acid (Figure 8E). In addition, when HA-tagged, activated Akt expressed in 293T cells was immunoprecipitated by an anti-HA antibody and incubated with hPP2A, exogenous Akt was also dephosphorylated by hPP2A in vitro (Figure 8E). These results demonstrated that E1A enhances PP2A activity through upregulation of the PP2A/C subunit and that PP2A can directly dephosphorylate Akt. Thus, PP2A may be involved in

regulation of the Akt/ASK1/p38 kinase cascade via dephosphorylation of Akt.

Considering the fact that p38 is known to activate PP2A phosphatase activity (Westermarck et al., 2001), these results also suggest that PP2A/Akt/ASK1/p38 may form a feed-forward loop to regulate cellular response to apoptosis (Figure 8F). To test the feed-forward regulatory mechanism, we asked whether blockage of Akt activation also activate PP2A activity. We found that blocking Akt activity using wortmannin indeed increased the PP2A activity and induced PARP cleavage while stimulating Akt activation by insulin-like growth factor 1 (IGF-1) repressed PP2A activity (Figure 8G). In addition, we observed that treatment with TNF-α, which has been shown to inhibit Akt phosphorylation (Figure 6D), also enhanced PP2A activity and PARP cleavage. However, the MEK1/2 kinase inhibitor PD98059 did not affect PP2A activity (Figure 8G). Blocking Akt activation using another PI3K inhibitor, LY249002, also increased PP2A activity (data not shown). These results support the feed-forward mechanism and showed that E1A, through upregulation of PP2A activity, may turn on this mechanism and induce sensitization to apoptosis.

Discussion

In the current study, we found that inactivation of the proapoptotic factor p38 accompanied by elevated expression of the oncogenic survival factor Akt is a common phenomenon in human cancer cells. The regulation between the Akt and p38 pathways is also a physiological phenomenon, as inactivation of Akt using either a gene knockout approach or chemical inhibitors resulted in elevated p38 activation, while activation of

Akt through either transfection of myristoylated, membrane-bound, constitutively active Akt or stimulation with IGF-1 results in p38 inactivation. The protein phosphatase PP2A was proposed to be involved in the integration of the Akt and p38 signaling pathways (Figure 8F). First, PP2A can dephosphorylate Akt, which inhibits Akt activity (Figure 8E). Also, because Akt can bind to ASK1 (Figure 8A) and phosphorylate ASK1 to inhibit ASK1 kinase activity (Gratton et al., 2001; Kim et al., 2001), dephosphorylation of Akt may enhance ASK1 kinase activity, resulting in elevated p38 phosphorylation (Figure 7A) (Ichijo et al., 1997; Moriguchi et al., 1996; Raingeaud et al., 1996; Tibbles et al., 1996). It has been shown that the p38 kinase can stimulate the phosphatase activity of PP2A (Westermarck et al., 2001). Thus, the enhanced PP2A activity again dephosphorylates Akt to inhibit Akt activity, and the PP2A/Akt/ASK1/p38 signal module can continue the feed-forward cycle to drive cells into a proapoptotic stage. This model is well consistent with all of the data presented in the current study and in the literature. For instance, PP2A directly dephosphorylated Akt (Figure 7F) and blockage of PP2A activity enhanced Akt phosphorylation while inhibiting p38 phosphorylation (Figure 7E). Also, inhibition of Akt activity increased p38 phosphorylation (Figures 7A, 7G, and 8A) and stimulated PP2A activity (Figure 7G), while activation of Akt by IGF-1 repressed PP2A activity (Figure 7G). Additionally, blocking p38 activity resulted in elevated Akt phosphorylation (Figure 7A). Compared with that in normal tissues and organs, the level of expression of phospho-Akt was dramatically elevated, while p38 activity was repressed in most of the human tumor tissues. The relative Akt and p38 activity may determine a cell's response to apoptotic stimuli, as they can be observed in E1A-mediated sensitization to apoptosis induced by serum starvation, TNF-α, UV-irradiation and

different categories of chemotherapeutic drugs (Figures 6B – 6D, and 7G). The results suggest that E1A, by increasing PP2A activity, turns on the feed-forward loop and reprograms the deregulated survival and death signals, which makes cells more sensitive to a variety of apoptotic stimuli.

The molecular pathways responsible for E1A-mediated chemosensitization include both the p53-dependent and -independent pathways (de Stanchina et al., 1998; Lowe, 1999; Putzer et al., 2000; Ries et al., 2000; Teodoro et al., 1995). Although the p53-dependent pathway, which requires functional p19ARF and p53, has been extensively characterized (Chiou et al., 1994; McCurrach et al., 1997; White, 1995), the p53-independent pathway has not been well defined. In the current study, we demonstrated that E1A can upregulate the PP2A/C subunit and turn on the feed-forward loop as proposed in Figure 8F to drive cancer cells to a stage that becomes much more sensitive to apoptosis. The p53-dependent mechanisms did not contribute to E1A-mediated sensitization to apoptosis in the current study, as both p53 and ARF were deleted in MDA-MB-231 cells. In wild type p53–expressing MCF-7 cells, however, the p53 protein level does not change in the presence or absence of E1A, and the ARF protein is undetectable (data not shown) (Stott et al., 1998). Thus, upregulating PP2A activity and reprogramming the deregulated survival and death signal to favor proapoptotic signaling may represent an alternative mechanism for p53-independent apoptotic sensitization by E1A.

Adenoviral type 5 E1A was originally described as an immortalization oncogene, primarily because of its ability to inactivate the tumor suppressor RB and collaborate with the ras or middle T oncogene to transform rodent primary culture cells (Harbour,

2000; Yu and Hung, 2000). However, E1A has also been found to be associated with multiple antitumor activities, including downregulation of Her-2/*neu* expression, induction of differentiation, upregulation of p19ARF, and p53 (de Stanchina et al., 1998; Frisch, 1996; Frisch, 2001a; Frisch, 2001b; Frisch and Mymryk, 2002; Ries et al., 2000; Ueno et al., 2000; Ueno et al., 1997; Yu and Hung, 2000). As a matter of fact, adenoviral type 5 E1A cannot transform established cell lines (Yu and Hung, 1998). It is interesting to note that recent data suggest that PP2A also plays an important role in human cancer (Mumby, 1995; Schonthal, 2001; Sontag, 2001). For example, mutations in the human PP2A regulatory subunit PP2A/A, either the α or β isoform, have been found in primary breast, colon, and lung tumors and melanoma, indicating that PP2A may act as a tumor suppressor (Calin et al., 2000; Sontag, 2001; Wang et al., 1998). In addition, recent evidence indicates that PP2A forms stable complexes with protein kinase–signaling molecules, and results of a gene knockout study of a catalytic subunit of PP2A, PP2A/C, indicate that it also is involved in signal transduction pathways controlling apoptosis (Gotz et al., 1998; Millward et al., 1999; Zolnierowicz, 2000). Furthermore, inactivation of PP2A may activate Akt and favor antiapoptotic signaling to contribute to the development of human cancer. In addition, a recent report suggested a tumor suppressor role for p38, as inactivation of p38 is involved in the development of human cancers by suppressing p53 activation (Bulavin et al., 2002). If this is the case, activation of PP2A and subsequent activation of p38 through repression of Akt by E1A may be another antitumor activity associated with E1A.

Experimental Procedures

Cell lines and cultures

HEK-293T cells and human breast cancer MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines 231-E1A and MCF-7-E1A were established as described previously (Meric et al., 2000). Akt1 knockout MEF cells and myristoylated, membrane-bound, constitutively active Akt1 (myr-Akt)–transfected stable Rat1 cells were provided by Dr. Nissim Hay (University of Illinois at Chicago, Chicago, IL) (Chen et al., 2001; Gottlob et al., 2001).

MTT assay

The standard MTT assay was performed as described previously (Ueno et al., 1997).

Transient transfection, luciferase assay, and FACS analysis

Expression vectors for HA-p38, CA-Akt, DN-Akt, and cytomegalovirus driving luciferase (pcDNA3-Luc) were used in this study. First, 1 x 10^5 cells in a 60-mm–well dish were transfected with 2.2 μg of total DNA using the DC-Chol cationic liposome as described previously (Ueno et al., 2000). After growing for 48 hr, the cells were split into three sets: one was used for a luciferase assay after exposure with or without Taxol for 24 hr, one was used to analyze Akt and p38 protein expression, and one was fixed in 75% ethanol, stained with propidium iodide (25 μg/ml), and sent for FACS analysis. The percentage of Taxol-treated cells that exhibited luciferase activity was normalized using the luciferase activity of the untreated cells as the baseline (100%). Standard deviations from three independent experiments were calculated.

Preparation of cell lysates, western blot analysis, and antibodies

Preparation of cell lysates and Western blot analysis were performed according to standard protocols. Information about the commercial antibodies used in this study is available upon request.

Immunoprecipitation

After transient transfection with HA-tagged p38 or CA-Akt, cells were stimulated using 10 μM insulin for 15 min. Cells were then lysed, and cell lysates were centrifuged at 14,000 rpm for 30 min. The supernatants were then transferred to a fresh tube. Proteins were cleared via addition of a normal mouse or rabbit IgG and immunoprecipitated with anti-p38, anti-Akt, or anti-HA antibodies. Immunoprecipitates were resolved by 10%

SDS-PAGE and transferred to nitrocellulose membranes. Akt, p38, and ASK1 were detected using Western blotting.

Kinase assay

Nonradioactive kinase assay kits for p38 and Akt were purchased from Cell Signaling (New England BioLabs, Beverly, MA). The p38 and Akt kinase activity were measured according to the manufacturer's protocol using GST-ATF-2 as the substrate for p38 and GST-GSK-3-β as the substrate for Akt.

Serine/threonine phosphatase assay

A nonradioactive serine/threonine phosphatase assay system was purchased from Promega Corporation (Madison, WI). PP2A phosphatase activity was measured according to the manufacturer's protocol.

Akt dephosphorylation assay and PP2A treatment

To measure endogenous Akt dephosphorylation by exogenous purified hPP2A (Upstate Biotechnnology, Lake Lacid, NY), cells were stimulated using 8 μM insulin (Sigma, St. Luis, MO) for 30 min before harvesting. Akt was immunoprecipitated, and aliquots of the Akt immunoprecipitates were incubated with various doses of hPP2A at 30°C for 30 min, after which the reaction was terminated through the addition of 6X SDS loading buffer and resolved using 10% SDS-PAGE. Phosphorylated and total Akt were measured using anti-phospho-Akt (Thr308-p) and total Akt, respectively. To analyze the dephosphorylation of ectopically expressed exogenous Akt, an HA-tagged, mimic

phosphorylated Akt construct (HA-Akt-DD) was transiently transfected into 293T cells, HA-tagged Akt was immunoprecipitated by an anti-HA monoclonal antibody, and a similar dephosphorylation reaction was performed as described for endogenous Akt. When required, 1 nM okadaic acid (OA) was added to the reaction mixture to block PP2A activity.

Establishment of IPTG-inducible DN-p38 stable cell lines

One E1A-expressing MDA-MB-231 clone was cotransfected with an IPTG-inducible DN-p38 construct (a gift from Philipp E. Schere, Albert Einstein College of Medicine, Bronx, NY) and the plasmid pCMVLacI (Stratagene, La Jolla, CA). Stable clones were selected in the presence of 200 μg/ml hygromycin.

Establishment of an orthotopic mammary tumor animal model and systemic E1A gene therapy in nude mice

MDA-MB-231 cells (1 x 10^6 cells/0.1 ml) or 231-E1A cells (2 x 10^6 cells/0.1 ml) in normal saline were subcutaneously injected into the mammary fat pad of female athymic 6- to 8-week old nu/nu mice (Charles River Lab.). Tumors were allowed to develop for 21 days; the mice were then randomly grouped and treated using the SN-liposome alone (SN, i.v.), Taxol alone (i.p., 10 mg/kg/injection, once a week over 6 weeks), E1A alone (i.v., 15 μg/mouse/injection, injection into mouse tail vein twice a week for 6 weeks), or E1A (i.v., 15 μg/mouse/injection) plus Taxol (i.p., 10 mg/kg/injection, 24 hr after the E1A injection over 6 weeks). Both the maximum and minimum diameter of the resulting tumors were measured twice a week using a slide caliper. Tumor volumes were

calculated by assuming a spherical shape for the tumor and using the formula, volume = $4/3r^3$, where r = one half of the mean tumor diameter measured in two dimensions. The mice were killed when the tumor was larger than 2 cm in diameter.

Immunohistochemistry, tissue microarray, and TUNEL assay

Tissue microarry slides (HistoArray #IMH-343/BA2 and IMH-304/CB2) were purchased from IMGENEX (San Diego, CA). Detailed information about each slide is available on-line at (http://www.imgenex.com/histoarrays/). Slide procession and immunohistochemistry staining were performed according to the manufacturer's protocol. Tissue–section preparation and TUNEL assay were performed as described previously (Hortobagyi et al., 2001; Hung et al., 2000; Yoo et al., 2001).

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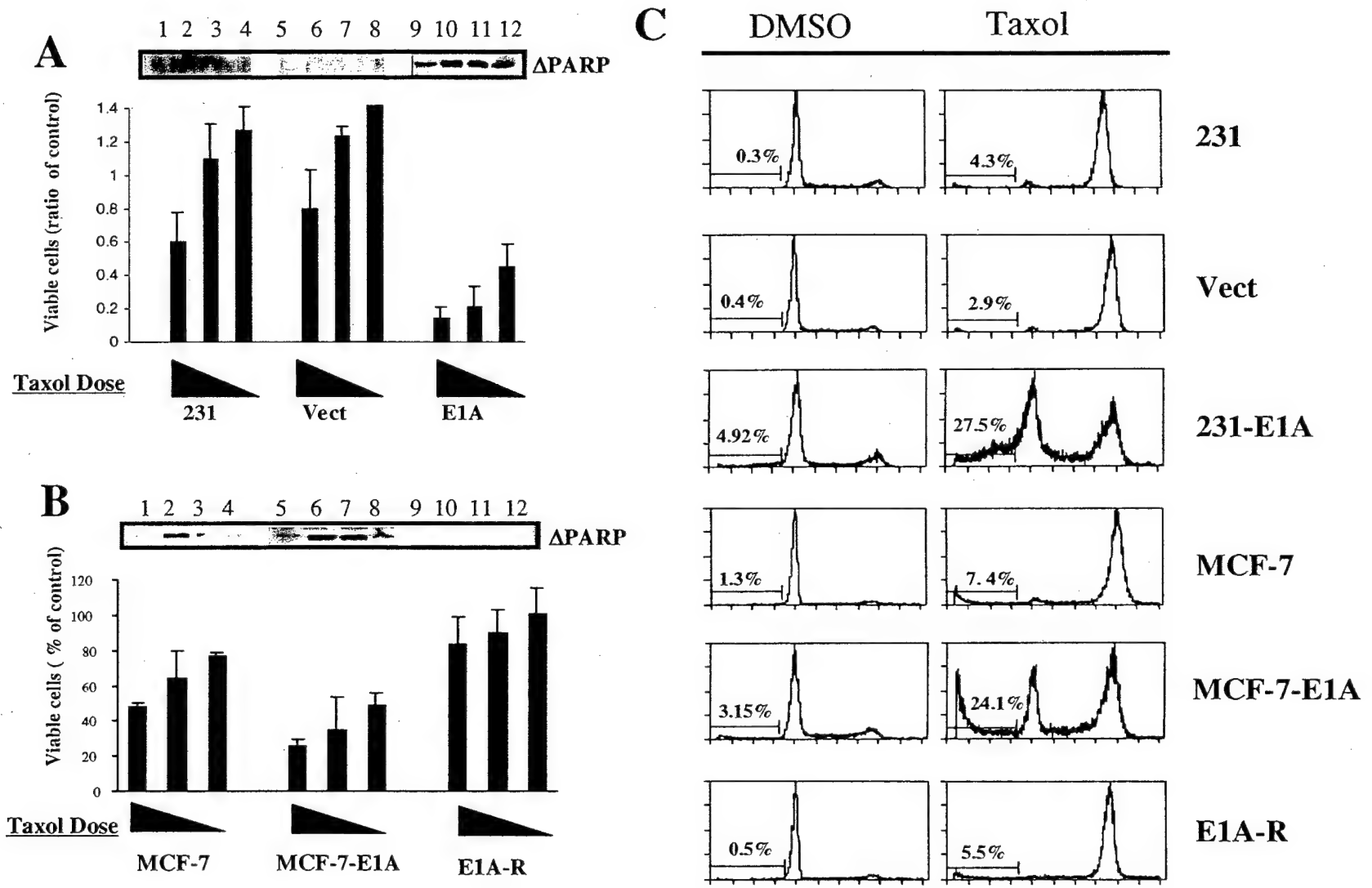
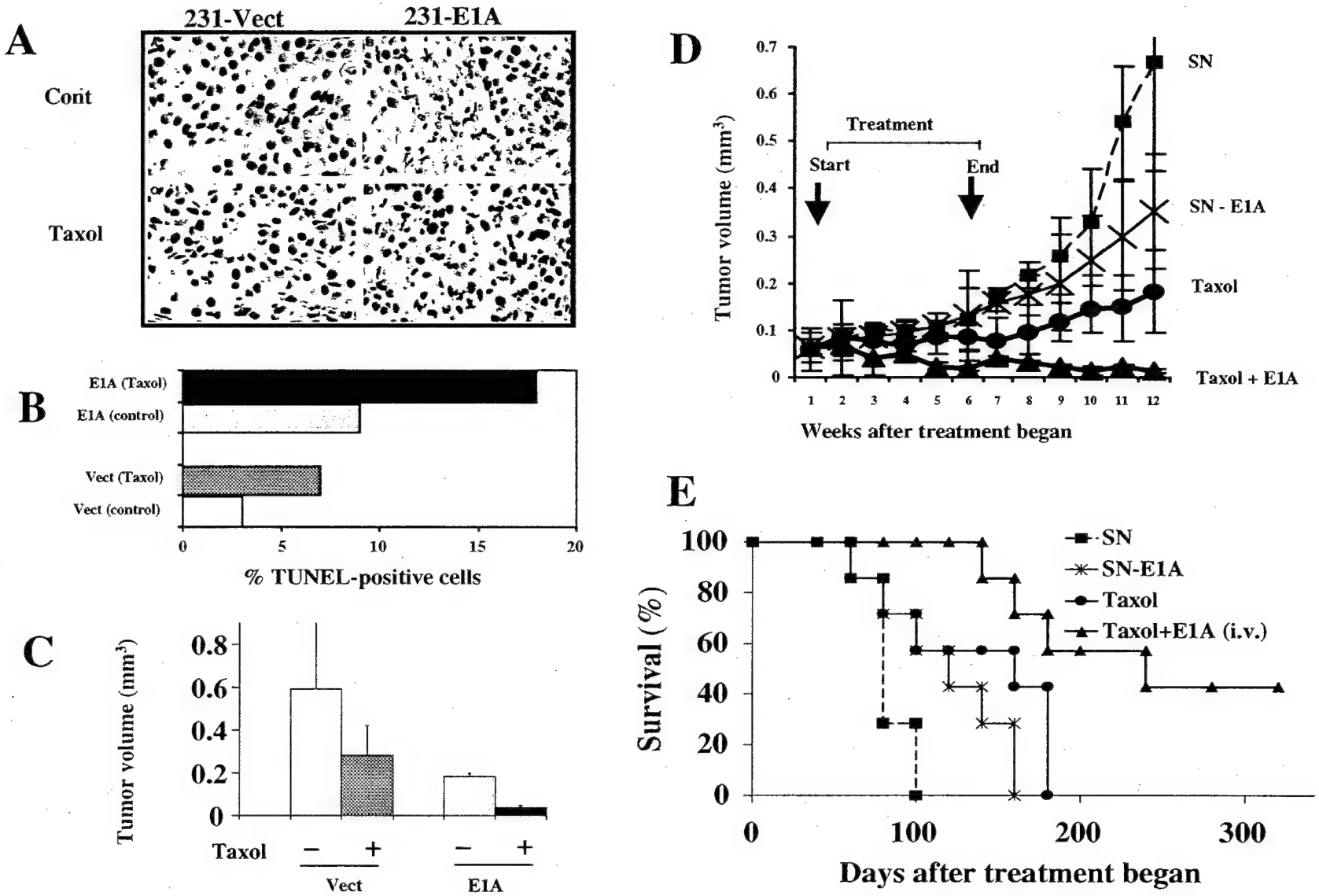
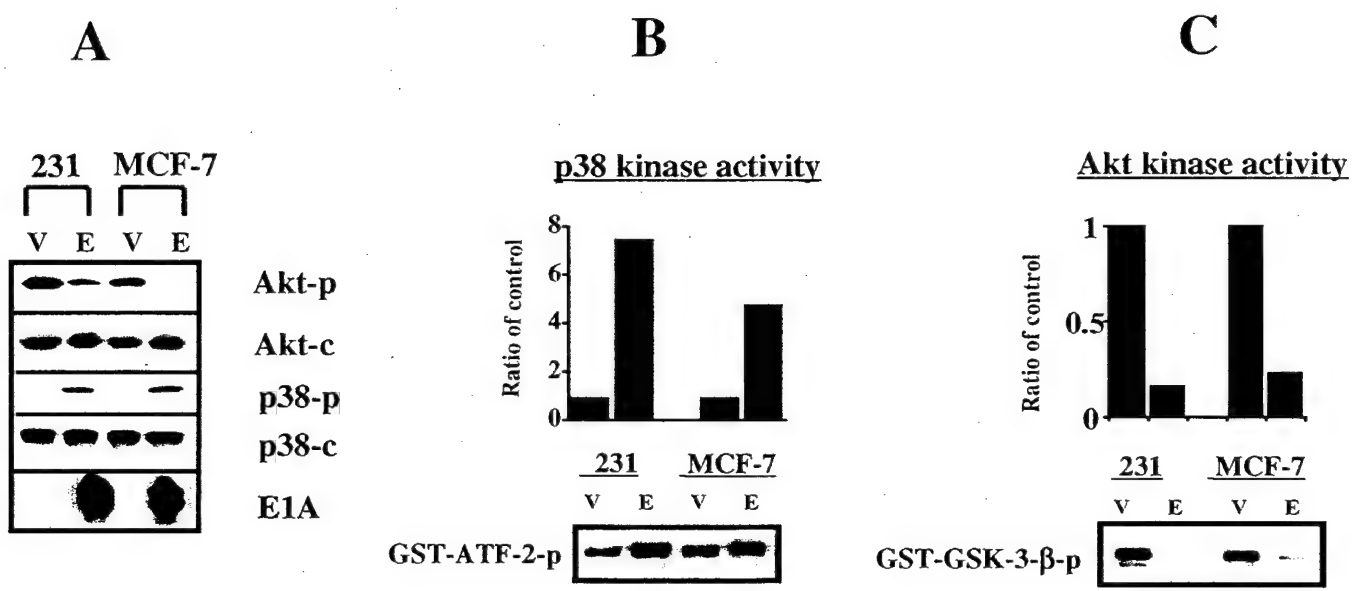
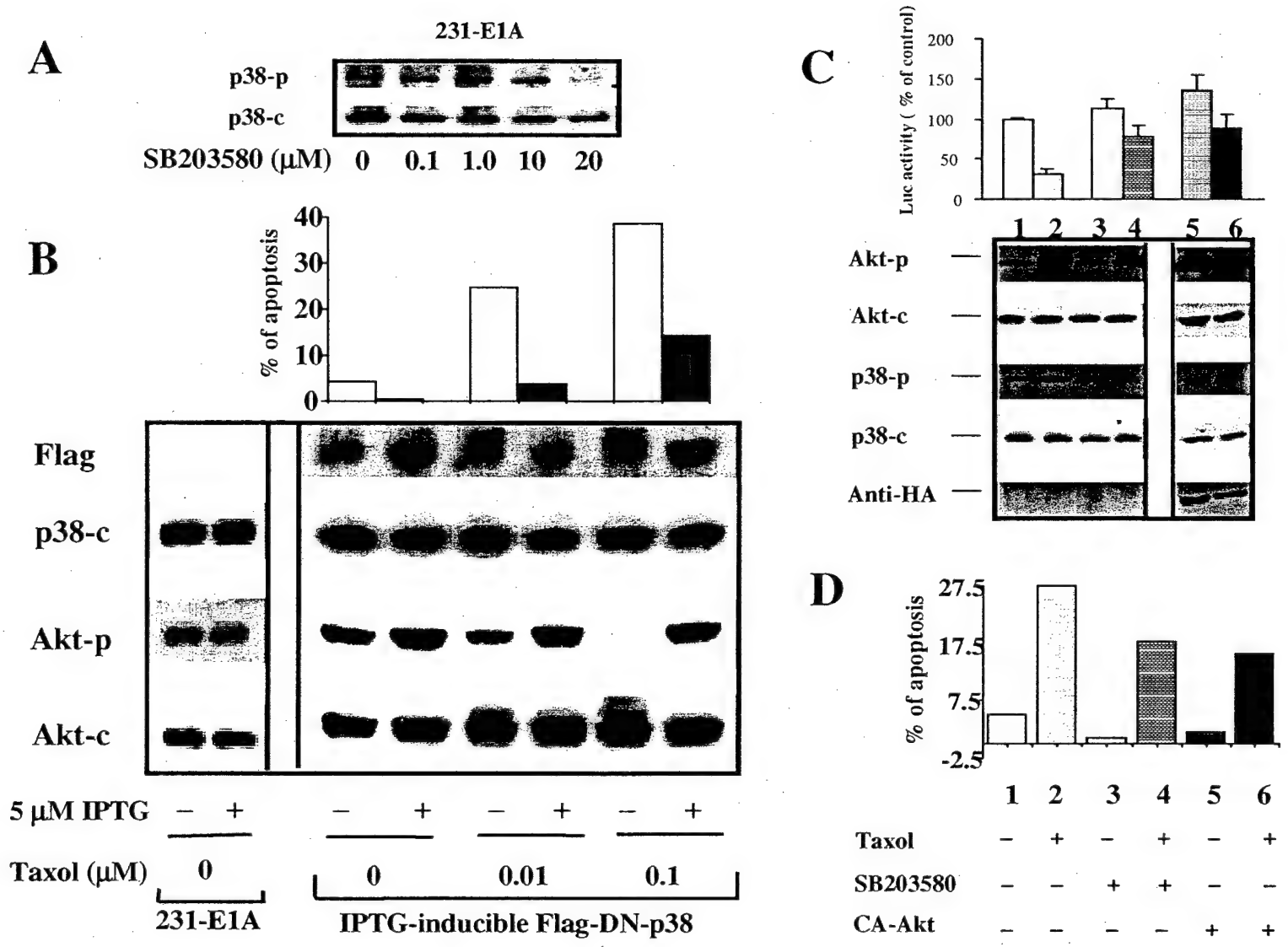
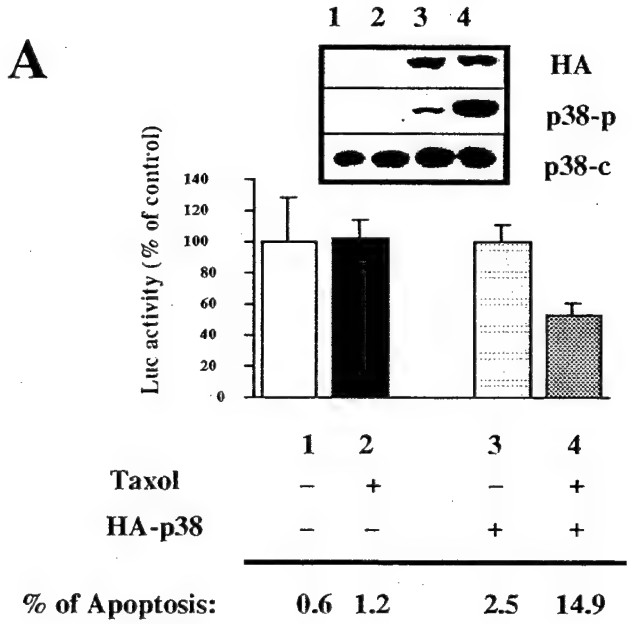
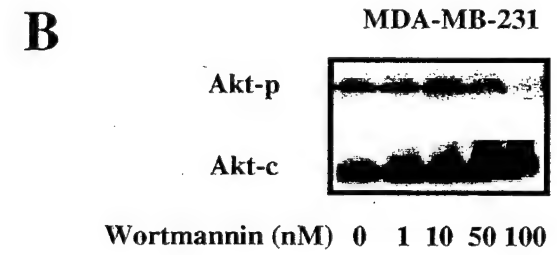

A
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (ratio of control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Taxol Dose
231
Vect
E1A
B
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (% of control)
120
100
80
60
40
20
0
Taxol Dose
MCF-7
MCF-7-E1A
E1A-R
C
DMSO
Taxol
0.3%
4.3%
231
0.4%
2.9%
Vect
4.92%
27.5%
231-E1A
1.3%
7. 4%
MCF-7
3.15%
24.1%
MCF-7-E1A
0.5%
5.5%
E1A-R

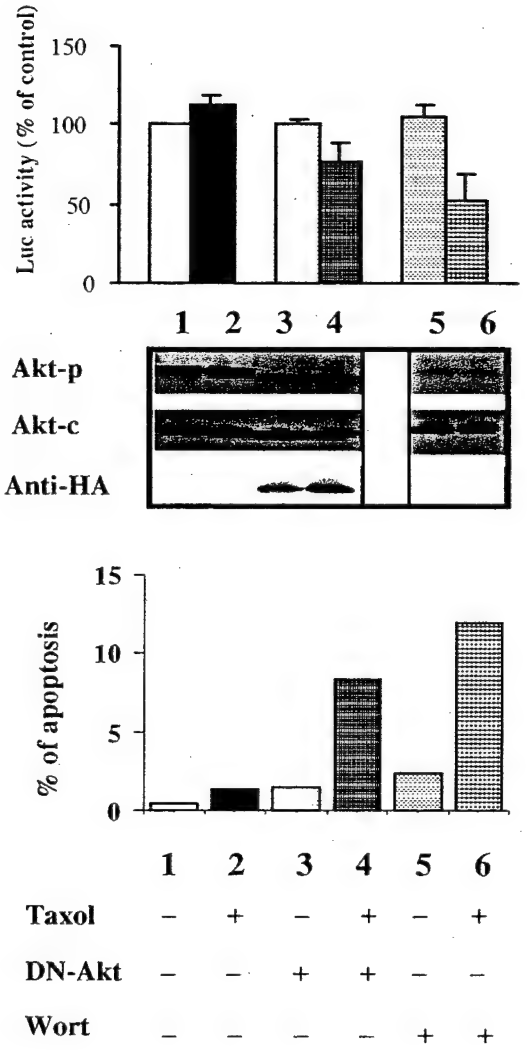

A
231-Vect
231-E1A
Cont
Taxol
B
E1A (Taxol)
E1A (control)
Vect (Taxol)
Vect (control)
0
5
10
15
20
% TUNEL-positive cells
C
Tumor volume (mm3)
0.8
0.6
0.4
0.2
0
Taxol
–
+
–
+
Vect
E1A
D
Tumor volume (mm3)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Treatment
Start
End
SN
SN - E1A
Taxol
Taxol + E1A
1
2
3
4
5
6
7
8
9
10
11
12
Weeks after treatment began
E
Survival (%)
100
80
60
40
20
0
SN
SN-E1A
Taxol
Taxol+E1A (i.v.)
0
100
200
300
Days after treatment began

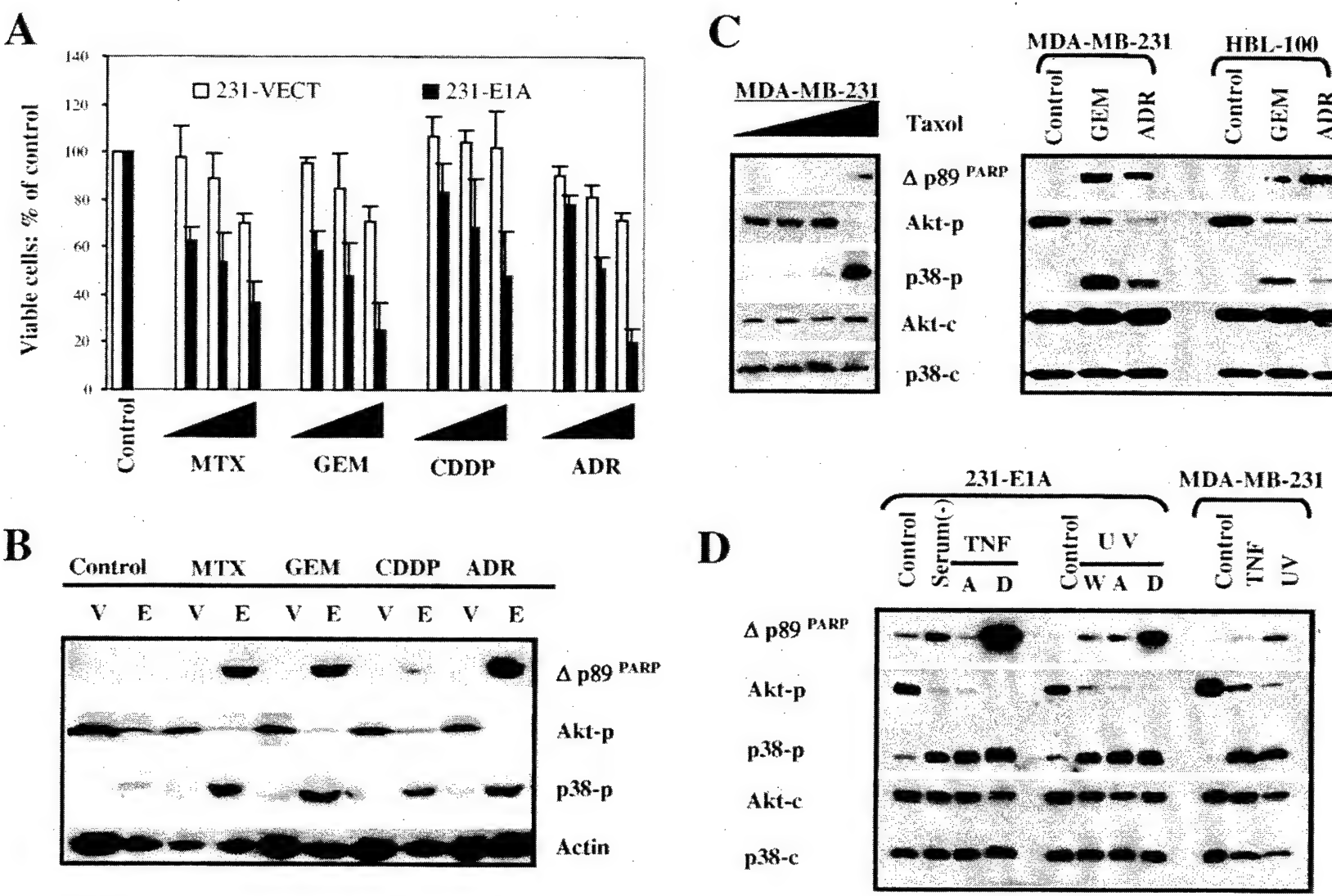

A
231
MCF-7
V E V E
Akt-p
Akt-c
p38-p
p38-c
E1A
B
p38 kinase activity
Ratio of control
8
6
4
2
0
231
MCF-7
V E V E
GST-ATF-2-p
C
Akt kinase activity
Ratio of control
1
0.5
0
231
MCF-7
V E V E
GST-GSK-3-β-p

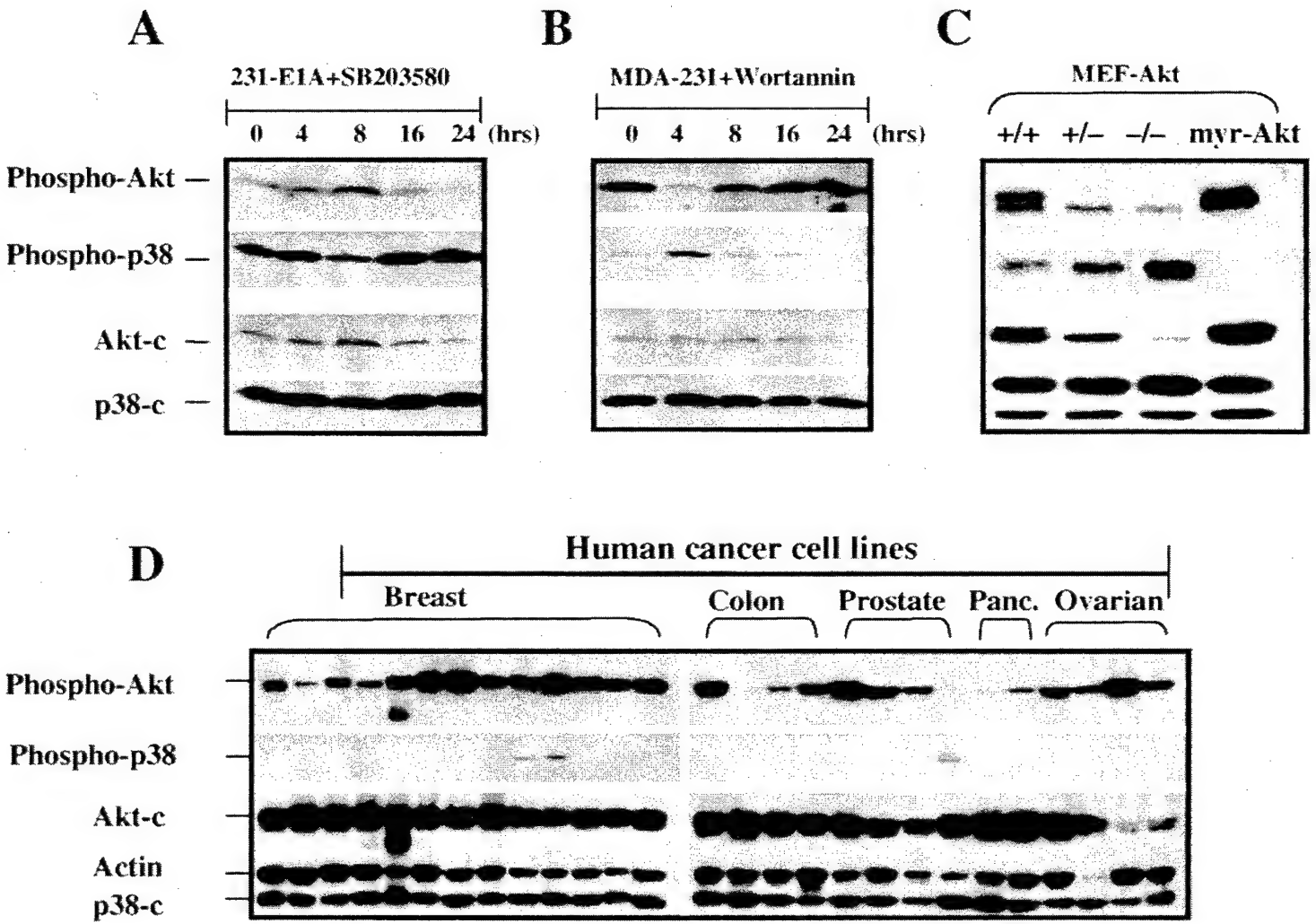

A
231-E1A
p38-p
p38-c
SB203580 (μM) 0 0.1 1.0 10 20
B
% of apoptosis
40
30
20
10
0
Flag
p38-c
Akt-p
Akt-c
5 μM IPTG – + – + – + – +
Taxol (μM) 0 0 0.01 0.1
231-E1A
IPTG-inducible Flag-DN-p38
C
Luc activity (% of control)
200
150
100
50
0
1 2 3 4 5 6
Akt-p
Akt-c
p38-p
p38-c
Anti-HA
D
% of apoptosis
27.5
17.5
7.5
-2.5
1 2 3 4 5 6
Taxol – + – + – +
SB203580 – – + + – –
CA-Akt – – – – + +


A
1 2 3 4
HA
p38-p
p38-c
Luc activity (% of control)
140
120
100
80
60
40
20
0
1 2 3 4
Taxol – + – +
HA-p38 – – + +
% of Apoptosis: 0.6 1.2 2.5 14.9

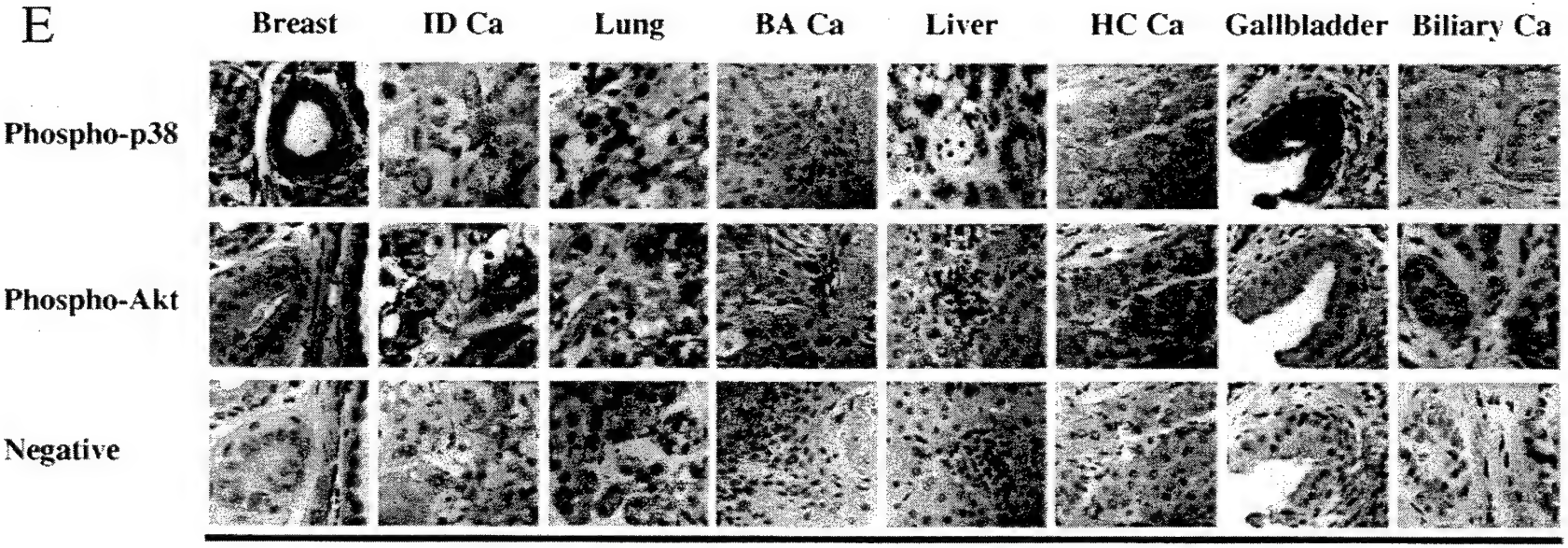

B
MDA-MB-231
Akt-p
Akt-c
Wortmannin (nM) 0 1 10 50 100


C
Luc activity (% of control)
150
100
50
0
1 2 3 4 5 6
Akt-p
Akt-c
Anti-HA
D
% of apoptosis
15
10
5
0
1 2 3 4 5 6
Taxol – + – + – +
DN-Akt – – + + – –
Wort – – – – + +


A
Viable cells: % of control
140
120
100
80
60
40
20
0
231-VECT
231-E1A
Control
MTX
GEM
CDDP
ADR
B
Control
MTX
GEM
CDDP
ADR
V E V E V E V E V E
Δ p89 PARP
Akt-p
p38-p
Actin
C
MDA-MB-231
Taxol
MDA-MB-231
HBL-100
Control
GEM
ADR
Control
GEM
ADR
Δ p89 PARP
Akt-p
p38-p
Akt-c
p38-c
D
231-E1A
MDA-MB-231
Control
Serum(-)
TNF
A D
Control
UV
W A D
Control
TNF
UV
Δ p89 PARP
Akt-p
p38-p
Akt-c
p38-c


A
231-E1A+SB203580
0 4 8 16 24 (hrs)
Phospho-Akt
Phospho-p38
Akt-c
p38-c
B
MDA-231+Wortannin
0 4 8 16 24 (hrs)
C
MEF-Akt
+/+ +/– –/– myr-Akt
D
Human cancer cell lines
Breast
Colon
Prostate
Panc.
Ovarian
Phospho-Akt
Phospho-p38
Akt-c
Actin
p38-c

E

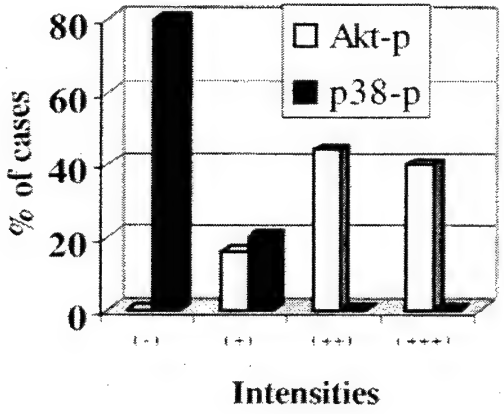


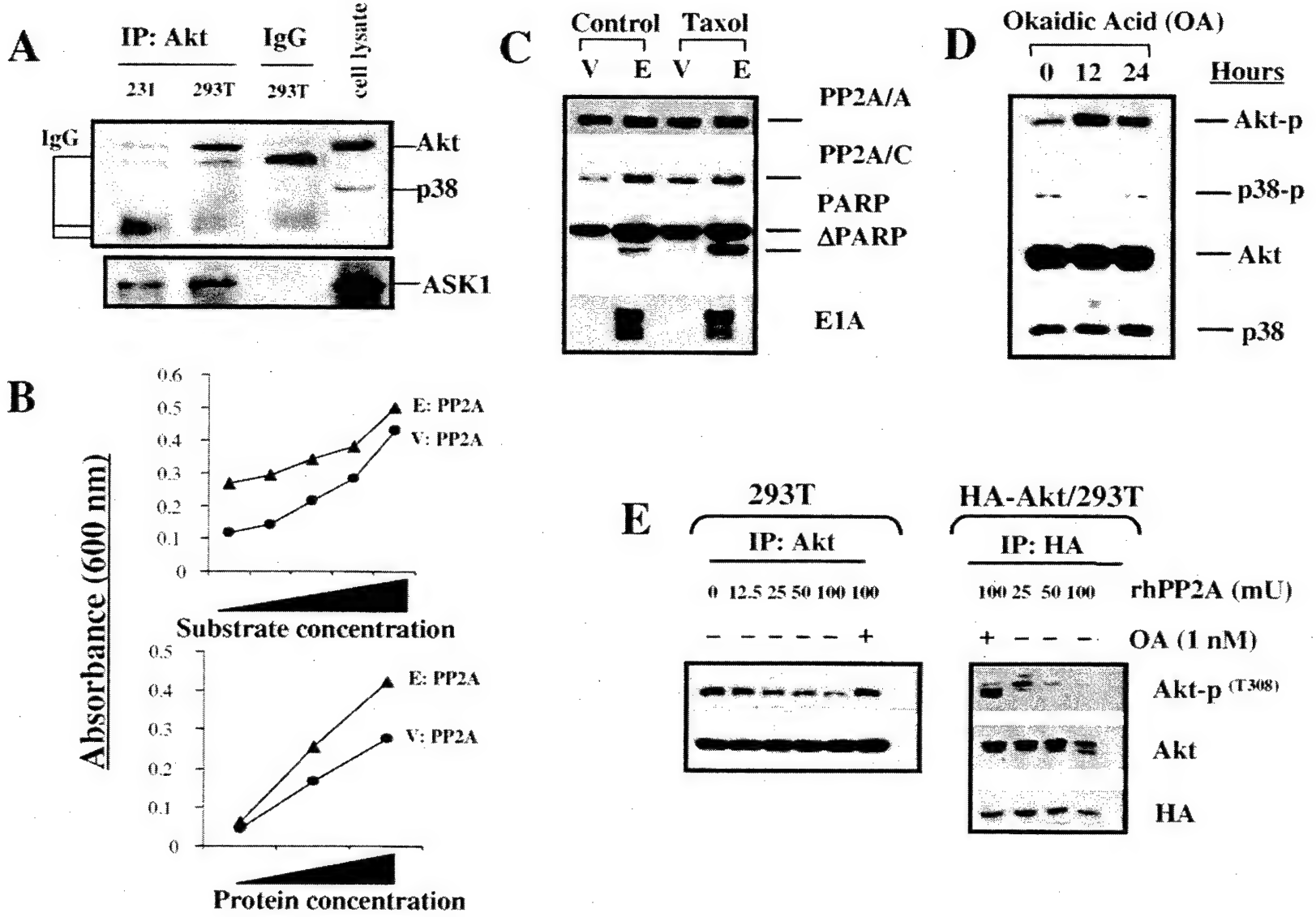
F

Immunohistochemistry analysis of phospho-Akt and phospho-p38 expression in human breast cancer tissues

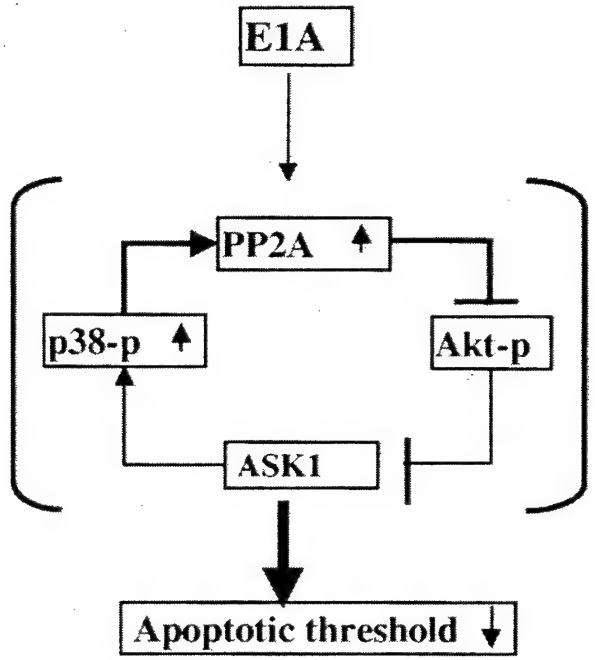
	Phospho-Akt		Phospho-p38	
T Stage	T1~T2 (n=25)	T3~T4 (n=25)	T1~T2 (n=25)	T3~T4 (n=25)
–	3 /25 (12%)	0 /25 (0)	18/25 (64%)	20/25 (80%)
+	11/25(44%)	4 /25 (16%)	8 /25 (32%)	5 /25 (20%)
++	6 /25 (24%)	11/25(44%)	1 /25 (4%)	
+++	6 /25 (24%)	10/25(40%)		

G




A
IP: Akt
IgG
231
293T
293T
cell lysate
IgG
Akt
p38
ASK1
B
Absorbance (600 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
E: PP2A
V: PP2A
Substrate concentration
0.5
0.4
0.3
0.2
0.1
0
E: PP2A
V: PP2A
Protein concentration
C
Control
Taxol
V
E
V
E
PP2A/A
PP2A/C
PARP
ΔPARP
E1A
D
Okaidic Acid (OA)
0
12
24
Hours
Akt-p
p38-p
Akt
p38
E
293T
IP: Akt
0 12.5 25 50 100 100
− − − − − +
HA-Akt/293T
IP: HA
100 25 50 100
rhPP2A (mU)
+ − − −
OA (1 nM)
Akt-p (T308)
Akt
HA

F


E1A
PP2A
p38-p
Akt-p
ASK1
Apoptotic threshold

G

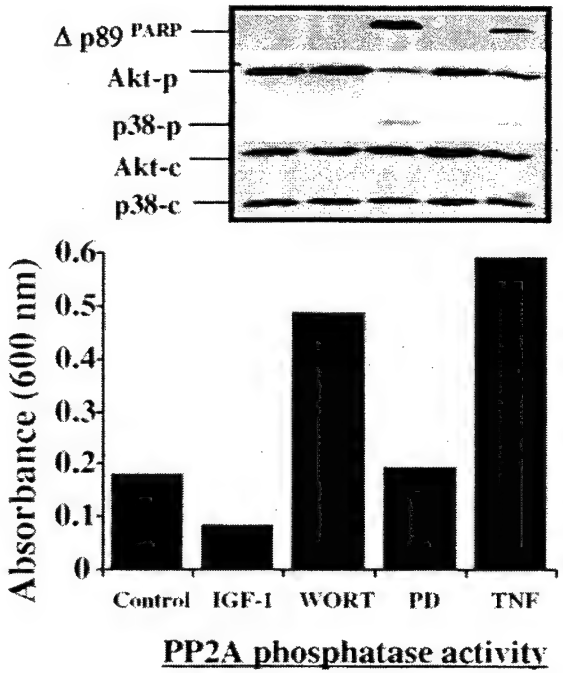

Δ p89 PARP
Akt-p
p38-p
Akt-c
p38-c
Absorbance (600 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
IGF-1
WORT
PD
TNF
PP2A phosphatase activity

Figure Legends

Figure 1. E1A–mediated sensitization to Taxol-induced apoptosis in vitro.

A: Percentage of viable cells after exposure to 0.1μM, 0.01 μM, and 0.001 μM of Taxol for 24 hrin MDA-MB-231 (231), vector-transfected cells (Vect), and E1A-expressing cells (E1A) detected using MTT assay. The number of viable cells without Taxol treatment was defined as 100%. The insert shows a cleaved PARP p89 fragment (ΔPARP) that was detected using a rabbit polyclonal antibody against cleaved PARP. Lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without Taxol treatment. **B**: Percentage of viable cells after exposure to Taxol in MCF-7 cells, MCF-7/E1A-expressing cells (MCF-E1A), and a revertant of E1A-expressing cells (E1A-R) detected using MTT assay. The insert shows ΔPARP, and lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without Taxol treamtment. **C**: FACS analysis of sub-G1–phase apoptotic cells with or without exposure to 0.01 μM Taxol. The symbols used are the same as those in panels A and B. DMSO, dimethyl sulfoxide.

Figure 2. Expression of E1A enhances Taxol anti-tumor effect in vivo.

A: TUNEL labeling. Cont, control. **B**: Percentage of apoptotic cells in tumor tissues with or without Taxol treatment. 231-Vect stable cells (1 x 10^6 cells/0.1 ml of NS/injection) and 231-E1A stable cells (~2 x 10^6 cells/0.1 ml of NS/injection) were inoculated into the mammary fat pad of mice. When tumors were established, the animals received Taxol (10 mg/kg, injected once a week over 6 weeks). **C:** Tumor volumes were measured, and tumor tissues from each group were collected and sectioned for detection of apoptotic cells (panel **A** and **B**). At least four animals were included in each group. The combination of systemic E1A gene therapy with Taxol-based chemotherapy enhance: **D**,

the anti-tumor effects of Taxol in nude mice and **E**, prolonged the animal's survival rate. Treatment groups included SN-liposome vehicle alone (■), SN-liposome-E1A (✶), Taxol alone (●), and Taxol plus SN-E1A (▲). At least seven animals were included in each group.

Figure 3. Upregulation of p38 activity and downregulation of Akt activity by E1A is correlated with E1A-mediated sensitization to Taxol-induced apoptosis.

A: Phospho-p38 (p38-p) and phospho-Akt (Akt-p) levels in E1A-expressing cells versus those in vector-transfected 231 and MCF-7 cells. Total p38 (p38-c) and Akt (Akt-c) were used as loading controls. **B** and **C:** Kinase assay of p38 and Akt and densitometric analysis of relative p38 activity using GST-ATF-2 as a substrate and Akt activity using GST-GSK-3-β as a substrate in E1A-expressing cells (E) versus vector-transfected cells (V).

Figure 4. Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to Taxol-induced apoptosis.

A: Dose-dependent effect of SB203580 on the phosphorylation of p38 in 231-E1A cells.

B: Repression of p38 activity by IPTG-inducible DN-p38 enhances Akt phosphorylation and abrogates E1A-mediated sensitization to Taxol in E1A-expressing cells. **C**: Western blot analysis of p38 and Akt in E1A expressing MDA-MB-231 cells and luciferase assay. The viability of cells with or without exposure to Taxol was measured using the luciferase activity. The pcDNA3-Luc vector was cotransfected into 231-E1A cells with (lanes 5 and 6) or without CA-Akt (lanes 1-4) before treatment using 0.01 μM Taxol. After exposure to Taxol for 4 hr, a portion of cells was harvested for protein extraction, while the rest were grown for 24 hr. D: The cells were then split into two portions: one

for a luciferase assay, and the other for FACS analysis to measure apoptosis.

Figure 5. Inceasing p38 or blocking Akt activity enhances cellular response to Taxol–induced apoptosis.

A: Luciferase assay of cells transiently transfected with HA-tagged p38 (lanes 3 and 4) and pcDNA3-Luc with or without exposure to 0.01 μM Taxol. The insert shows the expression of phospho-p38 and HA-tag in MDA-MB-231 cells. The percentage of cells undergoing apoptosis was determined using FACS analysis of sub-G1-phase cells after exposure to 0.01 μM Taxol for 24 hr (bottom). **B**: Dose-dependent effect of wortmannin on the phosphorylation of Akt in MDA-MB-231 cells. **C**: Western blot analysis of Akt and luciferase assay of cells transiently transfected with HA-tagged DN-Akt (lanes 3 and 4) or in the presence (lanes 5 and 6) or absence (lanes 1, 2) of Wortmannin. D: The percentage of cells undergoing apoptosis was obtained by FACS analysis of sub-G1-phase cells after exposure to 0.01 μM Taxol for 24 hr.

Figure 6. E1A-mediated sensitization to apoptosis induced by serum starvation, TNF-α, UV-irradiation, and different categories of anticancer drugs.

A: Percentage of viable cells in vector-transfected (231-Vect) and E1A-expressing MDA-MB-231 cells (231-E1A) after exposure to different doses of adriamycin (ADR; 0.1 μM, 1.0, and 10 μM), cisplatin (CDDP; 0.2, 2, and 10 μg/ml), gemcitabine (GEM; 0.2, 2, and 10 μg/ml), and methotrexate (MTX; 0.2, 2, and 10 μM) for 24 hr. Cell viability was measured using MTT assay. **B**: Downregulation of Akt phosphorylation and upregulation of p38 phosphorylation correlated with drug-induced PARP cleavage in stable 231-Vect (V) and 231-E1A (E) cells. The doses used were 1 μM ADR, 2 μg/ml CDDP, 2 μg/ml GEM, and 2 μM MTX. **C**: Dose-dependent effect of PARP cleavage and Akt and p38

phosphorylation in MDA-MB-231 and HBL-100 cells. The doses of Taxol used were ranging from 0, 0.001, 0.01, to 0.1 μM. The doses of GEM and ADR was 20μg/ml and 20 μM, respectively. **D:** Downregulation of Akt phosphorylation and upregulation of p38 phosphorylation correlated with PARP cleavage induced by serum starvation, TNF-α, and UV-irradiation. 231-E1A cells were serum–starved, exposed to TNF-α (5 ng/ml), or UV-irradiated (6 J/cm^2) for 20 hr, while parental MDA-MB-231 cells were exposed to 10X greater doses of TNF-α (50 ng/ml) and UV radiation (60 J/cm^2) for 20 hr. Both the attached cells and cells in suspension were collected, if not specified. W, whole cell lysate with both attached and suspended cells. A, attached cells only. D, detached or floated apoptotic cells.

Figure 7. Physiological regulation of the Akt and p38 pathways.

Stable E1A-expressing or parental MDA-MB-231 cells were serum-starved for 24 hr before exposure to **A:** 20.0 μM of SB203580 or **B:** 0.1 μM of Wartmannin. Cells were then harvested at indicated times, and proteins were extracted for Western blot analysis. **C**: Expression of phospho-p38 and phospho-Akt in Akt1 knockout MEF cells and myr-Akt–transfected Rat1cells and **D**: different types of cancer cell lines. Panc, pancreatic. **E**: Immunohistochemical staining of phospho-p38 and phospho-Akt in different types of human cancer tissues in the Histo-Array slides. Ca, carcinoma. ID Ca, infiltrating ductal carcinoma. BA Ca, bronchioloalveolar carcinoma. HC Ca, Hepatocellular carcinoma, Neg., Negative staining. **F**: Analysis of phospho-p38 and phospho-Akt expression in 50 cases of breast cancer at different stages. **G**: Inverse correlation between the intensity of phospho-Akt and phospho-p38 in stage III and IV breast cancer tissue samples.

Figure 8. Regulation of p38 and Akt phosphorylation through PP2A.

A: Immunoprecipitation and Western blot analysis of Akt and p38 interaction in 293T cells and MDA-MB-231 cells after transient transfection of HA-Akt and HA-p38. **B**: PP2A activity was measured using a protein phosphatase assay kit. The subtrate phospho-peptide concentrations were 100, 200, 500, 1,000, 1,500, and 2,000 μM, respectively. Protein concentrations include 0.1, 1.0, and 10 μg, respectively. V, 231-Vector. E, 231-E1A. **C**: Western blot analysis of PP2A and the catalytic subunit PP2A/C in 231-Vect (V) and 231-E1A (E) cells with or without exposure to 0.01 μM Taxol. **D**: Blocking PP2A activity by exposing 231-E1A cells to okadaic acid (10 nM) increased Akt phosphorylation and inhibited p38 phosphorylation. **E**: Direct dephosphorylation of Akt by purified hPP2A (1 mU = 1 x 10^{-3} U) and inhibition of by okadaic acid (OA; 1 nM) in vitro. **F**: A feed-forward model for the regulation of the p38 and Akt pathways. **G**: PP2A phosphatase activity and PARP cleavage in MDA-MB-231 cells with treatment using IGF-1 (50 ng/ml), TNF-α (50ng/ml), the PI3K inhibitor wortmannin (WORT; 0.5 μM), and the MEK1/2 inhibitor PD98058 (PD; 20 μM).

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Mark B. Wilson

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RE: *U.S. Patent Application No. 10/103,542 entitled "COMPOSITIONS AND METHODS FOR INACTIVATING THE AKT ONCOGENE AND/OR ACTIVATING THE P38 PRO-APOPTOTIC GENE" – Mien-Chie Hung and Yong Liao (Client reference: MDA00-059)*

Sir:

Please find enclosed:

(1) A Response to Notice to File Missing Parts of Nonprovisional Application filed under 37 C.F.R. 1.53(b) mailed April 15, 2002;

(2) Declaration;

(3) Power of Attorney;

(4) Copy of Notice to File Missing Parts of Nonprovisional Application filed under 37 C.F.R. 1.53(c)-Filing Date Granted;

(5) Check in the amount of $687.00; and

(6) A return postcard to acknowledge receipt of these materials. Please date stamp and mail this postcard.

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Respectfully submitted,

Mark B. Wilson
Reg. No. 37,259

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Yong Liao, Ph.D. (w/encl.)
David L. Parker, Esq. (w/o encl.)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Mien-Chie Hung
Yong Liao

Serial No.: 10/103,542

Filed: March 21, 2002

For: COMPOSITIONS AND METHODS FOR INACTIVATING THE AKT ONCOGENE AND/OR ACTIVATING THE P38 PROAPOPTOTIC GENE

Group Art Unit: 1646

Examiner: Unknown

Atty. Dkt. No.: UTSC:692US

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RESPONSE TO NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION FILED UNDER 37 C.F.R. 1.53(b)

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Sir:

In response to the Notice to File Missing Parts of Nonprovisional Application Under 37 C.F.R. § 1.53(b), dated April 15, 2002, there are enclosed herewith:

(a) Declaration executed on behalf of Mien-Chie Hung and Yong Liao;

(b) A Power of Attorney on behalf of Board of Regents, The University of Texas System;

(c) Our check in the amount of $687.00 to cover the basic filing fee ($370.00 - utility small entity); surcharge for late filing ($65.00); additional claims fee ($252.00); and

(d) A copy of Notice to File Missing Parts of Nonprovisional Application Filed Under 37 C.F.R. 1.53(b)-Filing Date Granted.

An Assignment to Board of Regents, The University of Texas System and a check for $40.00 are being filed under separate cover.

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Respectfully submitted,

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1996: 2nd Prize of Achievements in Liver Cancer Research awarded by the Funds of Zhao-You Tang &. Shanghai Construction Bank, Shanghai, P.R. CHINA
1995: Seeding Grant for Gene Therapy, Awarded by Shanghai Medical University
1995: Excellent Paper certified by the Shanghai Anti-Cancer Society during the Third Meeting of the Shanghai Anti-Cancer Society, Shanghai, 12/1/1995
1994: Excellent Graduate Student recognized by the Graduate School, Shanghai Medical University
1993: Winner of "Guang Hua Scholarship" due to excellent credits

Membership :

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Member, America Association for the Advancement of Science (AAAS)

Patent (pending):

Mien-Chie Hung and **Yong Liao**: Compositions and methods for inactivating the Akt oncogene and activating the p38 pro-apoptotic gene. (MDA00-050)
Applied for coverage under U.S. Patent (Serial No: 60/277,788 and Application No. 10/103.542), Chinese Patent (No. 011117869), and Taiwanese Counterpart Patent (N0. 90106723)

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HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation

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***HER-2/neu* amplification or overexpression can make cancer cells resistant to apoptosis and promotes their growth. p53 is crucial in regulating cell growth and apoptosis, and is often mutated or deleted in many types of tumour. Moreover, many tumours with a wild-type gene for p53 do not have normal p53 function, suggesting that some oncogenic signals suppress the function of p53. In this study, we show that *HER-2/neu*-mediated resistance to DNA-damaging agents requires the activation of Akt, which enhances MDM2-mediated ubiquitination and degradation of p53. Akt physically associates with MDM2 and phosphorylates it at Ser166 and Ser186. Phosphorylation of MDM2 enhances its nuclear localization and its interaction with p300, and inhibits its interaction with $p19^{ARF}$, thus increasing p53 degradation. Our study indicates that blocking the Akt pathway mediated by *HER-2/neu* would increase the cytotoxic effect of DNA-damaging drugs in tumour cells with wild-type p53.**

The *HER-2/neu* gene (also known as *c-erbB2*) encodes a 185-kDa transmembrane receptor tyrosine kinase that has partial homology with other members of the epidermal growth factor receptor family. Amplification or overexpression of *HER-2/neu* has been found in various cancer types and has been associated with poor clinical outcome, including short survival and short time to relapse[1–3]. We have previously showed that HER-2/*neu* activates the Akt pathway and so confers resistance to tumour necrosis factor (TNF)-induced apoptosis[4], and increases cell proliferation by inducing cytoplasmic localization of $p21^{Cip1/WAF1}$ (ref. 5). The phosphatidylinositol-3 kinase (PI(3)K)–Akt pathway plays an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy[6,7]. Several targets of the PI(3)K–Akt signalling pathway have recently been identified that might underlie the ability of this regulatory cascade to promote cell survival and cell growth. These substrates include two components of the intrinsic cell death machinery (Bad and caspase 9; refs 8,9), transcription factors of the forkhead family[10,11], Ikk-α (a kinase that regulates NF-κB activation[12]) and $p21^{Cip1/WAF1}$ (a cell-cycle inhibitor that controls cell growth[5]). The PI(3)K–Akt pathway has also been reported to delay p53-mediated apoptosis[13], suggesting that there is a link between the PI(3)K–Akt signalling pathway and p53-mediated apoptosis.

The tumour-suppresser protein p53 is a transcription factor that can induce either growth arrest or apoptosis and is frequently mutated or deleted in many types of tumour[14–21]. In addition, many tumours with a wild-type gene for p53 do not have functional p53 protein, suggesting that some oncogenic signals suppress the function of p53 (refs 14,17). The levels and activity of p53 are controlled largely by MDM2, which is amplified or overexpressed in a variety of human tumours and can function as an oncogene in tissue culture systems[14,17,20]. MDM2 can bind directly to p53 and promote its ubiquitination and subsequently its degradation by the proteasome[20,21]. The ability of MDM2 to degrade p53 depends on its ubiquitin E3 ligase activity and its nuclear localization signal (NLS) and nuclear export signal (NES), which are required for MDM2 to shuttle between the nucleus and the cytoplasm[14,20]. The nucleus–cytoplasm shuttling of MDM2 presumably mediates p53 degradation by cytoplasmic proteasomes.

In addition to MDM2, the transcriptional co-activator CBP/p300 has also been shown to play a role in efficient degradation of p53 (refs 22,23). CBP/p300 forms a complex with MDM2 *in vitro* and *in vivo*, and provides a platform to allow the assembly of the protein complex necessary for MDM2-mediated ubiquitination and degradation of p53 (ref. 23). MDM2 is feedback regulated by p53 and by the expression of ARF protein (p14 in humans and p19 in mice), which is encoded by the *Ink4A* locus[15,24–26]. Loss of ARF increases tumour susceptibility in mice, and mutations in *Ink4A* are often detected in human cancers[26]. The ARF protein binds directly to MDM2 to block p53 degradation by inhibiting the ubiquitin E3 ligase activity associated with MDM2 (ref. 27) and by sequestering MDM2 in nucleoli to prevent its export to the cytoplasm[28,29]. Interestingly, the regions that control MDM2 nucleus–cytoplasm shuttling (amino acids 181–185 of the NLS and amino acids 191–205 of the NES)[20] and the regions required for p300 binding (amino acids 102–222)[22,23] and $p19^{ARF}$ binding (amino acids 154–221)[24] overlap, suggesting that p300, $p19^{ARF}$ and other cellular proteins interact to control the function of MDM2.

In this study, we show that *HER-2/neu*-mediated resistance to DNA-damaging agents requires the activation of Akt, which enhances MDM2-mediated ubiquitination and degradation of p53. Akt interacts physically with MDM2 and phosphorylates it at Ser166 and Ser186, and so increases the nuclear localization of MDM2. Furthermore, phosphorylation of MDM2 increases its interaction with p300 and inhibits its interaction with $p19^{ARF}$ and so increases p53 degradation. Our study indicates that blocking the Akt pathway mediated by *HER-2/neu* would increase the cytotoxic effect of DNA-damaging drugs in tumour cells with wild-type p53.

Results

Blocking the Akt pathway sensitizes *HER-2/neu*-transformed cells to DNA-damaging agents. Because overexpression of *HER-2/neu* induces resistance to apoptosis mediated by chemotherapeutic drugs[3,4,30,31] and the Akt pathway is known to increase cell survival, we examined whether blocking the Akt pathway would sensitize cells to DNA-damaging drugs in our model system. This system

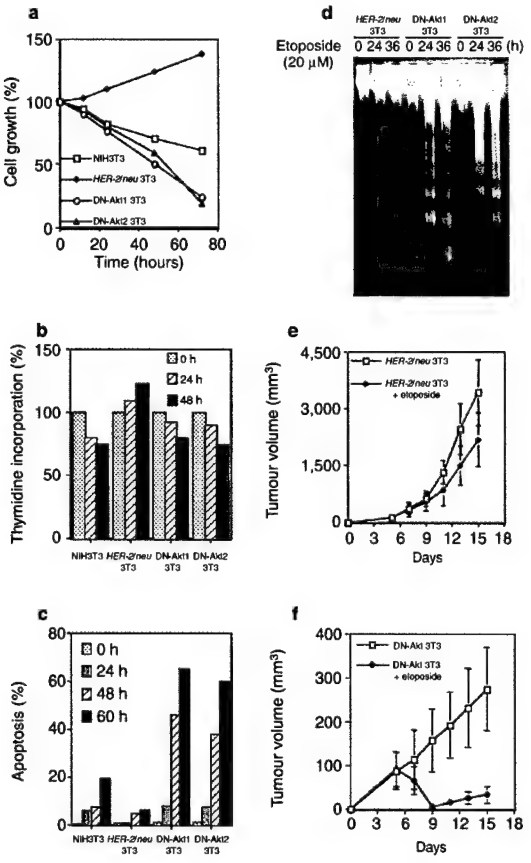

Figure 1 **The Akt pathway is required for *HER-2/neu*-mediated chemoresistance to etoposide. a**, Blocking the Akt pathway reduces the growth of DN-Akt 3T3 cells in the presence of etoposide. The cells (3×10^3) were seeded in 96-well plates and grown in Dulbecco's modified Eagle's medium/F12 medium plus 5% foetal bovine serum in presence of 20 μM etoposide. The growth rate was monitored by the MTT assay. The results are presented as the mean ± s.e.m. of three independent experiments performed in quadruplicate. **b**, Cells (3×10^3) were grown as described in (**a**) with 1 μCi of [^{3}H]-thymidine for 12 h. The cell replication rate was determined by measuring [^{3}H]-thymidine incorporation. The results are presented as the mean ± s.e.m. of three independent experiments performed in quadruplicate. **c**, Blocking the Akt pathway significantly induced apoptosis in DN-Akt 3T3 cells. The cells were grown in 5% serum plus 20 μM etoposide, and the percentage of cells in apoptosis was measured by fluorescence-activated cell sorting as described previously[4]. The results are presented as the mean ± s.e.m. of three independent experiments. **d**, Blocking the Akt pathway induced etoposide-mediated apoptosis in DN-Akt 3T3 cells, as measured by the DNA fragmentation assay[4]. The cells were grown in 100-mm plates as described in (**a**) with 20 μM etoposide. **e**, **f**, Tumours induced by DN-Akt 3T3 cells were much more sensitive to etoposide treatment. *HER-2/neu* 3T3 cells (**e**) and DN-Akt 3T3 cells (**f**) were inoculated subcutaneously in nude mice. One group of five mice from each cell line was treated with intravenous injections of etoposide on days 5 and 7 after the inoculation (solid symbols). The control groups (open symbols) were injected with the same volume of saline. The tumour volume was measured every two days, and the data represent the mean ± s.d. from five mice.

consists of NIH3T3 cells, *HER-2/neu* 3T3 cells (*HER-2/neu*-transformed NIH3T3 cells) and DN-Akt 3T3 cells (*HER-2/neu* 3T3 cells transfected with *DN-Akt*, a mutant *Akt* gene that produces a protein with no kinase activity)[4]. As expected, the *HER-2/neu* 3T3 cells were not affected by the DNA-damaging agent etoposide (Fig. 1). The Akt pathway is known to be constitutively activated in *HER-2/neu* 3T3 cells[4] and, when this pathway was blocked by DN-Akt in DN-Akt 3T3 cells, the cells grew much more slowly (Fig. 1a). When the DNA synthesis rate was determined by measuring [^{3}H]-thymidine incorporation, *HER-2/neu* 3T3 cells did not synthesize more DNA than the parental NIH3T3 cells or DN-Akt 3T3 cells (Fig. 1b).

The net cell growth rate depends on a fine balance between the cell proliferation rate and the cell death rate, and so we next examined whether apoptosis contributes to the difference in growth in these cells. There was significant difference in apoptosis among these cells as measured by fluorescence-activated cell sorting (FACS) analysis (Fig. 1c). The DN-Akt 3T3 cells underwent more apoptosis than did the *HER-2/neu* 3T3 cells after treatment with etoposide. The apoptosis in these cells was further confirmed by the DNA fragmentation in these cells (Fig. 1d). Therefore, the reduction in cell growth in DN-Akt 3T3 cells after treatment with etoposide was probably due to the increased apoptosis in these cells. Similarly, we treated these cells with another DNA-damaging agent, doxorubicin, and found that DN-Akt 3T3 cells were also more sensitive to apoptosis than *HER-2/neu* 3T3 cells (data not shown).

To examine whether the phenomenon can be observed *in vivo*, we inoculated *HER-2/neu* 3T3 cells and DN-Akt 3T3 cells into nude mice to generate mammary tumours, and tested the susceptibility of these tumours to DNA-damaging agents. Tumours from *HER-2/neu* 3T3 cells were not sensitive to etoposide (Fig. 1e), supporting the notion that *HER-2/neu*-overexpressing tumours are resistant to chemotherapeutic drugs, including DNA-damaging agents[3,30]. However, when the Akt pathway was blocked by DN-Akt, the tumours induced by DN-Akt 3T3 cells became very sensitive to etoposide (Fig. 1f). The dramatic tumour shrinkage after etoposide treatment is probably due to sensitization to apoptosis because massive apoptosis in these tumours was detected by the TUNEL assay (data not shown). Together, our results indicate that the Akt pathway is required for *HER-2/neu*-mediated drug resistance to DNA-damaging agents. Blocking this pathway sensitized *HER-2/neu*-overexpressing cells to DNA-damaging agents both *in vitro* and *in vivo*.

Activation of Akt-induced p53 ubiquitination and degradation. Because etoposide inhibits DNA topoisomerase II and causes DNA breaks, and because p53 is a sensor for damaged DNA and is induced by DNA damage[21,32], we tested whether p53 is involved in this sensitization effect mediated by the blockage of Akt pathway. We treated two pairs of cell lines ($p53^{-/-}$ and wild-type MEF cells, and p53-mutated MDA-MB453 and its DN-Akt/MDA453 transfectants) with etoposide. The p53-wild-type MEF cells were very sensitive to etoposide treatment (Fig. 2a). Blocking the Akt pathway using Wortmannin alone did not affect cell survival, but it dramatically enhanced the sensitization to etoposide treatment in p53-wild-type MEF cells (Fig. 2a). However, no effect was observed in the $p53^{-/-}$ cells (Fig. 2a). Similarly, blocking the Akt pathway with DN-Akt in p53-mutated DN-Akt/MDA453 cells could not sensitize the cells to etoposide treatment (Fig. 2b), although they did respond to TNF-induced apoptosis, as shown previously[4]. When similar experiments were performed in $p53^{-/-}$ and p53-wild-type cells, and apoptosis was measured by FACS analysis, we observed the same results (data not shown). Thus, these results suggest that p53 is crucial for etoposide-induced apoptosis.

To examine whether blockage of the Akt pathway would further sensitize p53-wild-type cells to etoposide-induced apoptosis in other cell types, we treated another two p53-wild-type (HBL-100 and MCF-7) and two p53-mutated (MDA-MB231 and SKOV3-ip1) cancer cell lines with etoposide. We found that the two p53-wild-type cell lines (HBL-100 and MCF-7) were more sensitive to apoptosis (as measured by FACS analysis) when the Akt pathway was blocked by a PI(3)K inhibitor (LY294002). However, in the two p53-mutated cells, the PI(3)K inhibitor could not sensitize the cells

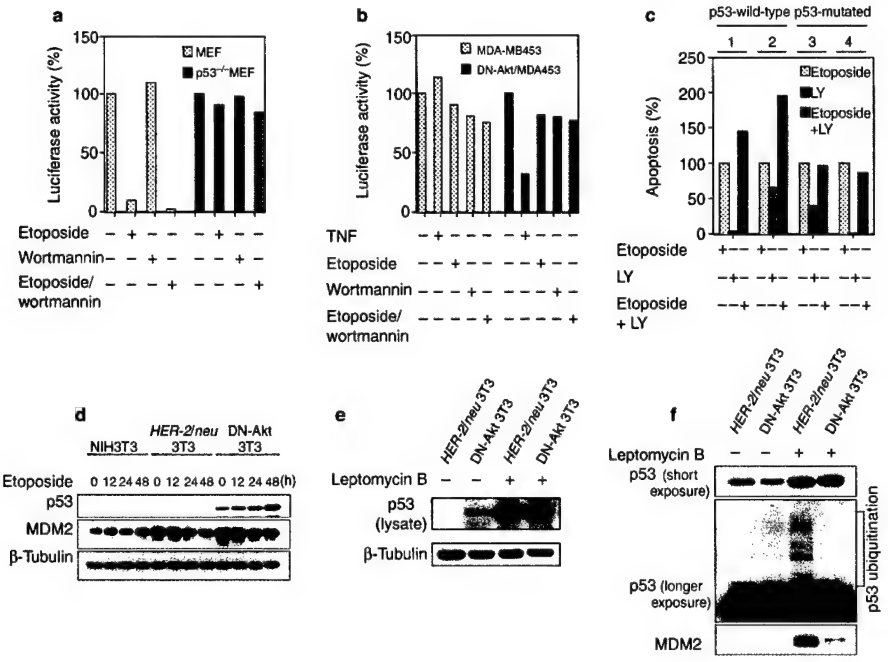

Figure 2 **Activation of Akt induced p53 ubiquitination and degradation.** **a**, The presence of p53 protein was required for sensitization to etoposide treatment. The p53-wild-type MEF and $p53^{-/-}$ MEF cells were transfected with 2 μg of a plasmid that contains actin-promoter-driven luciferase. After 12 h of transfection, cells were treated with 20 μM etoposide, 100 nM Wortmannin or 100 nM of Wortmannin plus 20 μM of etoposide and incubated for another 36 h. The cell lysates were prepared and the luciferase activity in each sample was measured. The sensitization to etoposide treatment was presented by the luciferase activity (mean ± s.e.m. in two separate experiments) over the control without any treatment. **b**, Blockage of the Akt pathway did not sensitize p53-mutated MDA-MB453 cells to etoposide treatment. Breast cancer cell line MDA-MB453 and its DN-Akt transfectants (DN-Akt/MDA453) cells were transfected with 2 μg of a plasmid that contains actin-promoter-driven luciferase. After 12 h of transfection, the cells were treated with 40 ng ml^{-1} of tumour necrosis factor, 20 μM etoposide, 100 nM of Wortmannin or 100 nM of Wortmannin plus 20 μM of etoposide as described in (**a**). The cell lysates were prepared and luciferase activity in each sample was measured. The sensitization to etoposide treatment was presented by the luciferase activity (mean ± s.e.m. in two separate experiments) over the control without any treatment. **c**, Blocking the Akt pathway sensitized p53-wild-type but not p53-mutated cells to etoposide-induced apoptosis. Two p53-wild-type (HBL-100 and MCF-7) and two p53-mutated (MDA-MB231 and SKOV3-ip1) cancer cell lines were treated with 20 μM etoposide in the presence or absence of the PI(3)K inhibitors LY294002 (50 μM) and Wortmannin (0.1 μM) (LY). Apoptotic cells in each sample were measured by flow cytometry using a fluorescence-activated cell sorter with propidium iodide staining. The two p53-wild-type cell lines were treated with etoposide and/or PI(3)K inhibitors for 24 h and harvested for analysis. Because few cells were found undergoing apoptosis in 24 h for the two p53-mutated cell lines, these two lines were treated with etoposide for 48 h to observe the apoptotic effect mediated by VP16. Apoptotic cells treated with etoposide alone in each sample were defined as 100%. Cell lines 1–4 were HBL-100, MCF-7, MDA-MB231 and SKOV3-ip1, respectively. **d**, Blockage of the Akt pathway led to production of p53. NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with 20 μM etoposide, for the indicated times. Whole cell lysates (50 μg) were prepared and subjected to western blot analyses with antibodies specific for p53 (Ab-7), MDM2 and β-tubulin. **e**, Blocking nuclear export induced p53 stabilization in *HER-2/neu* 3T3 and DN-Akt 3T3 cells. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with 5 ng ml^{-1} leptomycin B for 7 h or not treated, and the p53 and β-tubulin levels were analysed by western blotting. **f**, Activation of Akt induced the ubiquitination of p53 and increased the binding of MDM2 to p53. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were treated with leptomycin B as in (**e**) or left alone. The cell lysates (1.2 mg of *HER-2/neu* 3T3 protein and 0.4 mg of DN-Akt 3T3 protein) were immunoprecipitated with monoclonal anti-p53 antibody (Ab-1) and the precipitates were then subjected to western blotting with polyclonal anti-p53 antibody (Ab-7) for a short (10 sec) or a longer (1 min) exposure. The same membrane was used again for western blotting against MDM2.

to etoposide-induced apoptosis (Fig. 2c). Similar results were also obtained when we used another PI(3)K inhibitor, Wortmannin (data not shown). Therefore, sensitization to etoposide-induced apoptosis occurred only in p53-wild-type cells, not in p53-mutated or $p53^{-/-}$ cells, suggesting the involvement of p53 and Akt in the etoposide-induced apoptosis.

We next examined the expression of p53 in NIH3T3, *HER-2/neu* 3T3 and DN-Akt 3T3 cells before and after treatment with etoposide. We did not observe the induction of p53 expression in NIH3T3 and *HER-2/neu* 3T3 cells, but did find time-dependent induction of p53 in DN-Akt 3T3 cells (Fig. 2d). Surprisingly, basal expression of p53 in DN-Akt 3T3 cells was dramatically elevated (Fig. 2d), indicating that blocking the Akt pathway increases the p53 protein level. The half-life of p53 is ~15 min, and p53 is present at low levels in cells and only increases in response to physiological stress such as DNA damage[21,32]. The p53 protein level increases as a result of post-translational modification, which stabilizes the protein and is largely controlled by MDM2. MDM2 can bind to p53, promote its ubiquitination and shuttle it to cytoplasm, where it is subsequently degraded by the proteasome. Leptomycin B (LMB), a cytotoxin produced by streptomycetes, can block nuclear export and stabilize p53 accumulated in the nucleus of LMB-treated cells[33]. To rule out the possibility that the lack of expression of p53 in *HER-2/neu* 3T3 cells was due to a genetic defect in these cells, we treated the cells with LMB. The concentration of p53 increased in *HER-2/neu* 3T3 cells after treatment with

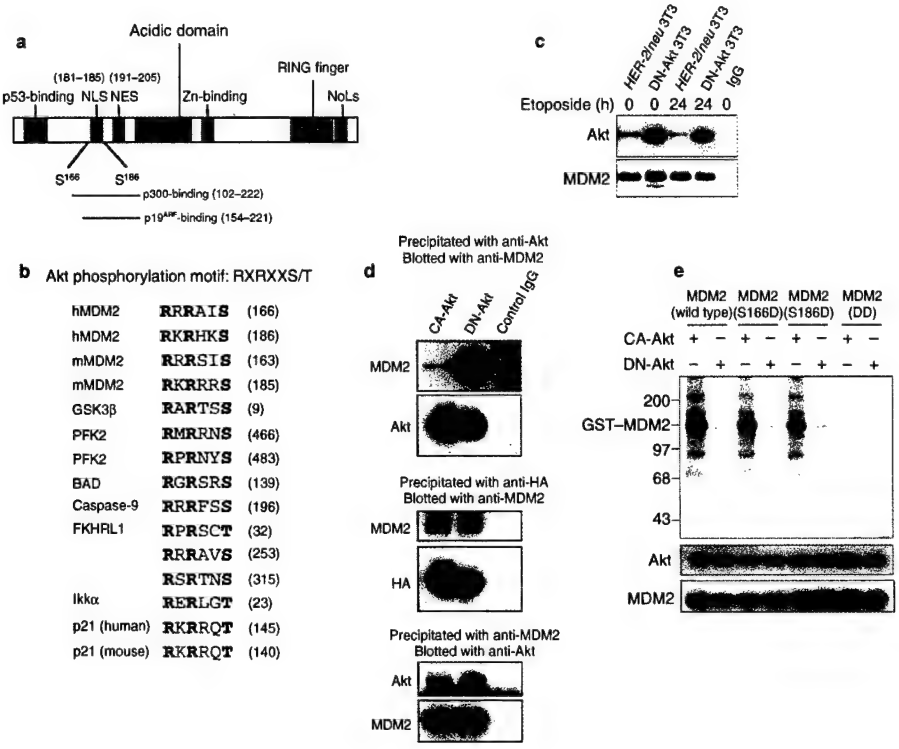

Figure 3 **Akt interacts with MDM2 and phosphorylates Ser166 and Ser186.** **a**, Structure of the MDM2 protein, showing the positions of the nuclear-localization signal (NLS), the nuclear-export signal (NES) and domains that interact with p300 and $p19^{ARF}$. **b**, The consensus Akt phosphorylation motif is highlighted. Sequences of MDM2 and other known Akt substrates are shown for comparison. **c**, Immunoprecipitation of endogenous MDM2 and detection of endogenous Akt. Endogenous MDM2 was immunoprecipitated from 1000 μg of *HER-2* 3T3 or DN-Akt 3T3 cell lysates with an MDM2-specific antibody (SMP14) or control IgG. After transfer to a nitrocellulose membrane, endogenous Akt was detected with an Akt-specific antibody (Upstate Biotechnology). **d**, Association between Akt and MDM2. HA-tagged DN-Akt or CA-Akt (10 μg) was transfected into 293T cells by the calcium phosphate method. The cells were lysed in RIPA buffer after 48 h and Akt was immunoprecipitated with anti-Akt (Upstate Biotechnology) or ani-HA (HA.11; Babco) antibody. After transfer to a nitrocellulose membrane, MDM2 was detected with MDM2-specific antibody. Alternatively, the cell lysates were immunoprecipitated with MDM2-specific antibody. After transfer to a nitrocellulose membrane, the bound Akt was detected with Akt-specific antibody. **e**, Akt phosphorylated MDM2 at Ser166 and Ser186. HA-tagged CA-Akt or DN-Akt (20 μg) was transiently transfected into 293T cells as described in Methods. After 48 h of incubation, CA-Akt and DN-Akt were immunopricipitated with an anti-HA antibody and incubated with 5 μg of either GST–wild-type MDM2 or GST–mutant MDM2 (S166D, S186D and S166,186DD) in a kinase buffer containing 5 μCi of [γ^{32}P]ATP for 30 min at 30 °C. The kinase reaction was stopped by the addition of SDS-PAGE buffer and the samples were assayed by autoradiography. The lower two panels show western blots for Akt and GST–MDM2 used in the phosphorylation reaction, detected with antibodies against Akt and MDM2, respectively.

LMB (Fig. 2e). These results indicate that the absence of p53 in *HER-2/neu* 3T3 cells was not caused by a genetic defect but rather by the destabilization of p53 in these cells.

To compare the ubiquitination of p53 in these cells, we used more cell lysate from *HER-2/neu* 3T3 cells than from DN-Akt 3T3 cells to produce equal amounts of immunoprecipitated p53 before and after treatment with LMB. Surprisingly, there was dramatically more ubiquitination of p53 in *HER-2/neu* 3T3 cells than in DN-Akt 3T3 cells (Fig. 2f). The difference in ubiquitination of p53 was not due to a difference in p53 levels, because nearly equal amounts of p53 were immunoprecipitated from *HER-2/neu* 3T3 and DN-Akt 3T3 cells (short exposure in Fig. 2f). When the same membrane was analysed by western blot using MDM2-specific antibody, we found more MDM2 binding to p53 immunoprecipitated from *HER-2/neu* 3T3 cells (Fig. 2f). Thus, the increased ubiquitination of p53 observed in *HER-2/neu* 3T3 cells resulted largely from increased binding of MDM2 to p53 in these cells (Fig. 2f). These results indicate that the activation of Akt could increase the binding of MDM2 to p53 and induce the ubiquitination of p53. Blocking the Akt pathway stabilized the p53 protein by inhibiting the binding of MDM2 to p53, consequently suppressing the ubiquitination and degradation of p53.

Akt interacts with and phosphorylates MDM2. The inability of MDM2 to bind and ubiquitinate p53 in DN-Akt 3T3 cells seems to be responsible for the cell's increased p53 level and sensitization to DNA-damaging agents. Recently, MDM2 was found to be phosphorylated by ATM (for ataxia-telangiectasia mutated), disrupting its binding to p53 and leading to the stabilization of p53 (ref. 34). We noticed that there were two potential Akt phosphorylation sites (Ser166 and Ser186) in the MDM2 protein (Fig. 3a), and that these two sites were highly conserved across species (Fig. 3b). These two potential phosphorylation sites were in the regions crucial for MDM2 nuclear import and export, and required for p300 and $p19^{ARF}$ binding. To determine whether Akt interacts with and phosphorylates MDM2, and so regulates its function, we performed immunoprecipitation experiments to detect the association

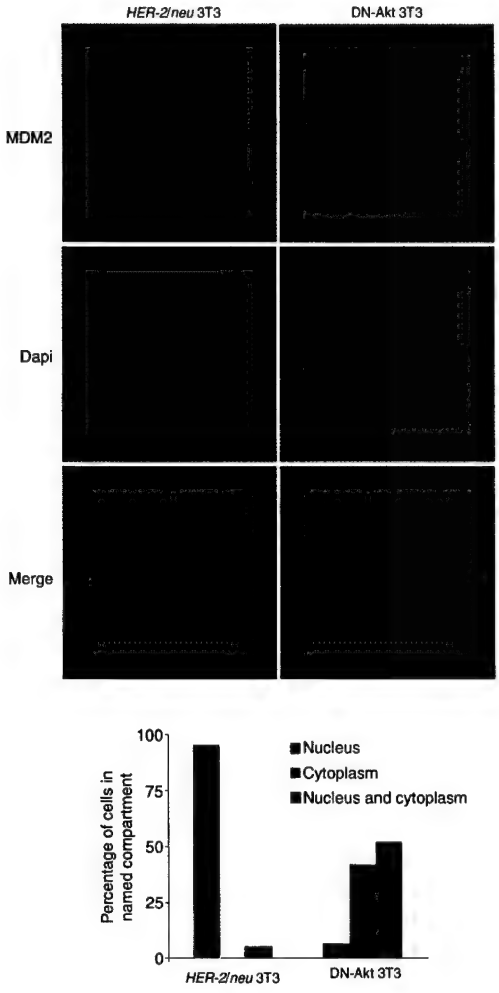

Figure 4 **Akt affects the cellular localization of endogenous MDM2.** **a**, ~2×10⁴ *HER-2* 3T3 and DN-Akt 3T3 cells were plated into chamber slides for 16 h. After fixation of the samples in 4% paraformaldehyde for 1 h and permeabilization with 0.2% Triton X-100 for 30 min, the cellular localization of MDM2 was detected using a monoclonal antibody against MDM2 (SMP14). After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG plus DAPI and examined under a fluorescent microscope (Zeiss). **b**, Quantification of subcellular MDM2 staining. The subcellular localizations of MDM2 in *HER-2* 3T3 and DN-Akt 3T3 cells were assessed in 100 cells in several different views. The graph shows the percentages of cells with the indicated MDM2 subcellular localization.

between Akt and MDM2. In an immunoprecipitation of endogenous MDM2, we detected the presence of endogenous Akt (Fig. 3c). The binding of Akt to MDM2 was increased in DN-Akt 3T3 cells (Fig. 3c). Treatment with etoposide does not seem to affect the interaction between MDM2 and Akt. We also transfected 293T cells with haemagglutinin (HA)-tagged constitutively active Akt (CA-Akt) or DN-Akt. After immunoprecipitating Akt or HA, we detected MDM2, and vice versa (Fig. 3d), indicating that these two molecules are associated. MDM2 seems to associate more strongly with DN-Akt than with CA-Akt.

To examine whether Akt can phosphorylate MDM2, we transfected CA-Akt and DN-Akt into 293T cells and immunoprecipitated Akt from cell lysates. After incubation with glutathione-*S*-transferase (GST)–MDM2 (wild-type), GST–MDM2 (S166D), GST–MDM2 (S186D) or GST–MDM2 (S166, 186DD) in a kinase reaction mixture, we found that Akt phosphorylated MDM2 at Ser166 and Ser186 with equal efficiency, whereas DN-Akt could not (Fig. 3e). When both Ser166 and Ser186 were mutated to aspartic acid, CA-Akt could not phosphorylate this double mutant of MDM2, suggesting that Ser166 and Ser186 in MDM2 were specific for Akt phosphorylation (Fig. 3e). The fact that no phosphorylation was observed in the double mutant of MDM2 (S166,186DD) was not due to the lack of Akt kinase or MDM2 proteins, because nearly equal amount of kinase and protein were used (Fig. 3e, bottom two panels). These results indicate that Akt physically interacted with MDM2 and phosphorylated MDM2 at Ser166 and Ser186.

Akt induces nuclear localization of MDM2. Because the Akt phosphorylation sites in MDM2 are close to the NLS and NES, we examined whether the activation of Akt affects the cellular localization of MDM2. We performed immunofluorescence staining to analyse the cellular localization of endogenous MDM2 in *HER-2/neu* 3T3 and DN-Akt 3T3 cells. In the parental *HER-2/neu* 3T3 cells, MDM2 was usually found in the nucleus (95% of cells), with little in the cytoplasm (Fig. 4a). However, in DN-Akt-transfected cells, MDM2 was either in the cytoplasm (42%) or distributed evenly between the cytoplasm and nucleus (50%) (Fig. 4b). MDM2 has been previously reported to localize mainly to the nucleus. The inability of MDM2 to localize to the nucleus in DN-Akt 3T3 cells suggests that phosphorylation of MDM2 by Akt is required for its nuclear translocation. Blocking the Akt pathway in DN-Akt 3T3 cells would inhibit the nuclear import of MDM2, and so result in the stabilization of p53.

We next tested whether the phosphorylation of MDM2 by Akt affects the cellular localization of MDM2. We transfected $p53^{-/-}$ and $MDM2^{-/-}$ MEF cells with CA-Akt or DN-Akt together with wild-type or mutant MDM2 and examined the cellular localization of MDM2 by immunofluorescence analysis. As shown in Fig. 5, wild-type MDM2 was found predominantly in the nucleus in the presence of CA-Akt but was localized to both the cytoplasm and the nucleus when DN-Akt was introduced. Mutants of MDM2 (S166D, S186D or S166,186DD, in which serine 166 or/and serine 186 were replaced with aspartic acid to mimic the phosphorylation of MDM2 by Akt) were found predominantly in the nucleus even in the presence of DN-Akt. These results strongly indicate that Ser166 and Ser186 of MDM2 are crucial in determining the cellular localization of MDM2, and that the phosphorylation of Ser166 and Ser186 of MDM2 by Akt resulted in the nuclear localization of MDM2. Taken together, our results indicate that Akt can interact with MDM2 and phosphorylate the Ser166 and Ser186 residues in MDM2, leading to the nuclear localization of MDM2.

These results establish the regulation of MDM2 by the HER-2/*neu*–Akt pathway in cell culture. To examine whether this phenomenon also existed in tumour tissues, we compared the levels of activated (phosphorylated) Akt and the cellular localization of MDM2 in 33 cases of human breast tumours that are either HER-2/*neu* and Akt positive or HER-2/*neu* and Akt negative by immunostaining. In 21 HER-2/*neu* and Akt positive breast tumour tissues, we found that MDM2 was mainly present in the nucleus. By contrast, in all 12 HER-2/*neu* and Akt negative breast tumour tissues examined, MDM2 was localized in both the nucleus and cytoplasm (Fig. 6). Similar results were obtained when we used another MDM2 antibody (NCL-MDM2; Novocastra Laboratories, Newcastle upon Tyne, UK) (data not shown). The subcellular localization of MDM2 and the activation of Akt were significantly correlated based on the analysis using Fisher's exact test (Table 1). Thus, the correlation between these two molecules in primary tumour samples was consistent with our *in vitro* data from cell lines and transfection studies (Figs 4, 5). The tumour staining data agree

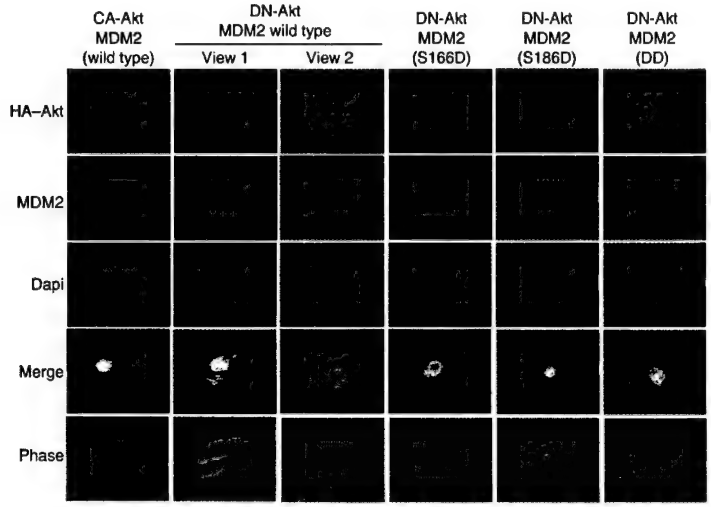

Figure 5 **Akt affects the subcellular localization of MDM2.** A 1:1 ratio of CA-Akt or DN-Akt (2 μg) and the wild-type or mutant MDM2 (2 μg) were transfected into $p53^{-/-}$ and $MDM2^{-/-}$ MEF cells. After 36 h of incubation, the cells were trypsinized and plated into chamber slides for another 12 h. After fixation, the cellular locations of CA-Akt, DN-Akt and MDM2 were detected using antibodies against HA (HA.11, Babco) and MDM2 (Ab-2, Oncogene Research). After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG plus Dapi, and examined under a fluorescent microscope (Zeiss). When wild-type MDM2 was transfected with CA-Akt, MDM2 were found in the nucleus in ~95% of the transfected cells. However, when MDM2 was transfected with DN-Akt, 25% of the transfected cells had MDM2 staining only in the nucleus and 75% of the cells had MDM2 staining in both the nucleus and the cytoplasm. When the S166D, S186D or S166,186DD mutant of MDM2 was transfected with DN-Akt, ~90% of the cells had MDM2 staining only in the nucleus.

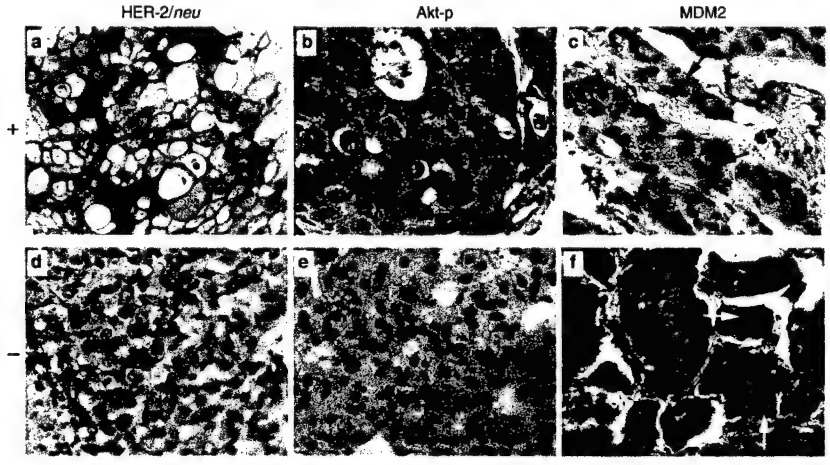

Figure 6 **HER-2/*neu* activates Akt and induces nuclear localization of MDM2 in breast tumour tissues.** Tissue sections from the HER-2/*neu*- and Akt-positive adenocarcinomas (**a–c**) and HER-2/*neu*- and Akt-negative adenocarcinomas (**d–f**) were stained with antibodies specific to HER-2/*neu* (**a** and **d**), phosphorylated Akt (**b** and **e**), MDM2 (**c** and **f**) or normal rabbit serum (data not shown). The immunostaining was visualized with peroxidase-conjugated secondary antibody. The arrows indicate the nuclear and cytoplasmic localization of MDM2 in **c** and **f**, respectively.

with the observation in cell culture and further strengthen the hypothesis that overexpression of *HER-2/neu* can regulate the cellular distribution of MDM2 via the activation of Akt.

Phosphorylated MDM2 induces preferential binding to p300 but not to $p19^{ARF}$. Because the Akt phosphorylation sites Ser166 and Ser186 in MDM2 were also within the region required for MDM2 to bind p300 and $p19^{ARF}$, we investigated whether the loss of p53 ubiquitination in DN-Akt 3T3 cells was due to changed binding of MDM2 to p300 and $p19^{ARF}$. We immunoprecipitated MDM2 from *HER-2/neu* 3T3 or DN-Akt 3T3 cells and examined

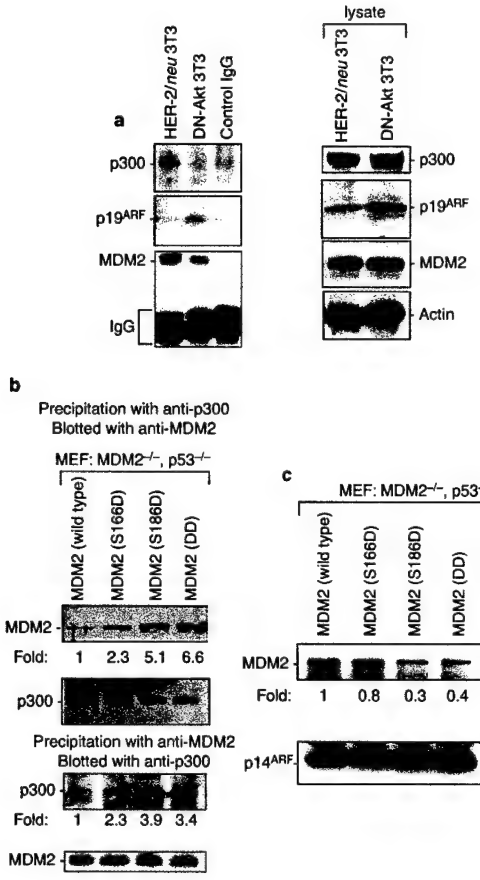

Figure 7 **Phosphorylated MDM2 binds differently to p300 and p19^{ARF}.** **a**, MDM2 from *HER-2/neu* 3T3 and DN-Akt 3T3 cells had different binding affinities to p300 and p19^{ARF}. Cell lysates from *HER-2/neu* 3T3 or DN-Akt 3T3 cells were immunoprecipitated with monoclonal anti-MDM2 antibody (SMP14) and the precipitates were then subjected to western blotting analysis for the binding of p300 and p19^{ARF}. The same lysates were used for western blotting analysis for the expression of p300, p19^{ARF}, MDM2 and actin. **b**, Preferential binding of the phosphorylated form of MDM2 to p300. The wild-type and phosphorylated forms of MDM2 mutants were transfected into p53$^{-/-}$ and MDM2$^{-/-}$ cells. After 48 h of culture, the cell lysates were immunoprecipitated with anti-p300 and anti-MDM2 antibodies, and the precipitates were subjected to western blot analysis for the binding of MDM2 and p300. The intensities of the bands were quantified by densitometry using NIHimage and are shown under the western blots. **c**, Weaker binding of phosphorylated form of MDM2 to p14^{ARF}. The wild-type and phosphorylated forms of MDM2 were transfected with p14^{ARF} into p53$^{-/-}$ and MDM2$^{-/-}$ cells. After 48 h of culture, the cell lysates were immunoprecipitated with antibodies against p14^{ARF} and the precipitates were subjected to western blot analysis for the binding of MDM2. The intensity of the bands was quantified by densitometry using NIHimage and are shown under the western blot.

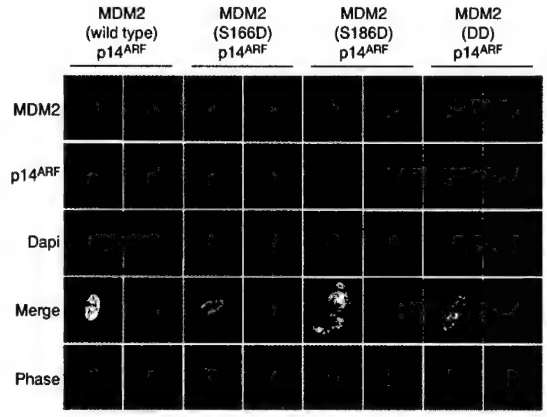

Figure 8 **Nuclear localization of wild-type and mutant MDM2 is not affected by the presence of p14^{ARF}.** A 1:1 ratio of wild-type or mutant MDM2 (2 μg) and p14^{ARF} (2 μg) were transfected into p53$^{-/-}$ and MDM2$^{-/-}$ MEF cells. After 36 h of incubation, the cells were trypsinized and plated into chamber slides for another 12 h. After fixation, the cellular localization of MDM2 and p14^{ARF} were detected by using antibodies against MDM2 (SMP14) and p14^{ARF} (Ab-1; Neomarkers, Fremont, California), respectively. After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit plus Dapi and examined under a fluorescent microscope (Zeiss). For each transfection staining, cells producing both MDM2 and p14^{ARF} are shown on the left-hand side and cells expressed only p14^{ARF} are shown on the right-hand side.

MDM2's association with p300 and p19^{ARF}. We found that MDM2 from *HER-2/neu* 3T3 cells bound much more strongly to p300 than in DN-Akt 3T3 cells (Fig. 7a). However, p19^{ARF} binding to MDM2 was dramatically lower in *HER-2/neu* 3T3 cells than in DN-Akt 3T3 cells (Fig. 7a). The differences in p300 and p19^{ARF} bound by MDM2 between *HER-2/neu* 3T3 and DN-Akt 3T3 cells were not due to a difference in protein levels, because equal amounts of MDM2 were immunoprecipitated from these cells and the expression of p300 and p19^{ARF} in these cells was the same (Fig. 7A). We also transfected wild-type MDM2 and phosphorylated forms of MDM2 (S166D, S186D or S166,186DD) into MDM2$^{-/-}$ and p53$^{-/-}$ cells, and analysed the binding of these phosphorylated forms of MDM2 with p300. As seen in Fig. 7b, after immunoprecipitating the p300, the association of phosphorylated forms of MDM2 to p300 was stronger than that of wild-type MDM2. Similarly, when we immunoprecipitated the wild-type and phosphorylated forms of MDM2, we found that more p300 bound to the phosphorylated forms of MDM2. These results indicate that Akt can phosphorylate MDM2 at Ser166 and Ser186, and that the phosphorylated MDM2 has a stronger binding with p300 than the unphosphorylated form.

We also transfected wild-type MDM2 and phosphorylated forms of MDM2 (S166D, S186D or S166,186DD) into MDM2$^{-/-}$ and p53$^{-/-}$ cells with p14^{ARF}, and analysed the binding of these phosphorylated forms of MDM2 with p14^{ARF}. As shown in Fig. 7c, slightly less of the associated phosphorylated forms of MDM2 were present after immunoprecipitating the p14^{ARF} than of wild-type MDM2. The ARF protein binds directly to MDM2 to block p53 degradation by inhibiting the ubiquitin E3 ligase activity associated with MDM2 (ref. 27) and by sequestering MDM2 in nucleoli to prevent its export to the cytoplasm. There is persuasive evidence that ARF can inhibit the ubiquitin ligase activity of MDM2 *in vitro*, but exactly how ARF affects the nucleus–cytoplasm shuttling of MDM2 remains a matter of debate[15]. Current models propose that ARF function depends on its ability to sequester MDM2 in the nucleolus[15]. We examined whether the nucleoplasmic or nucleolus localization was different in wild-type and mutant MDM2 proteins. We transfected p53$^{-/-}$ and MDM2$^{-/-}$ MEF cells with p14^{ARF} together with wild-type or mutants of MDM2, and examined the nucleus and nucleolus localization of MDM2 by immunofluorescence analysis. As shown in Fig. 8, wild-type MDM2 was found

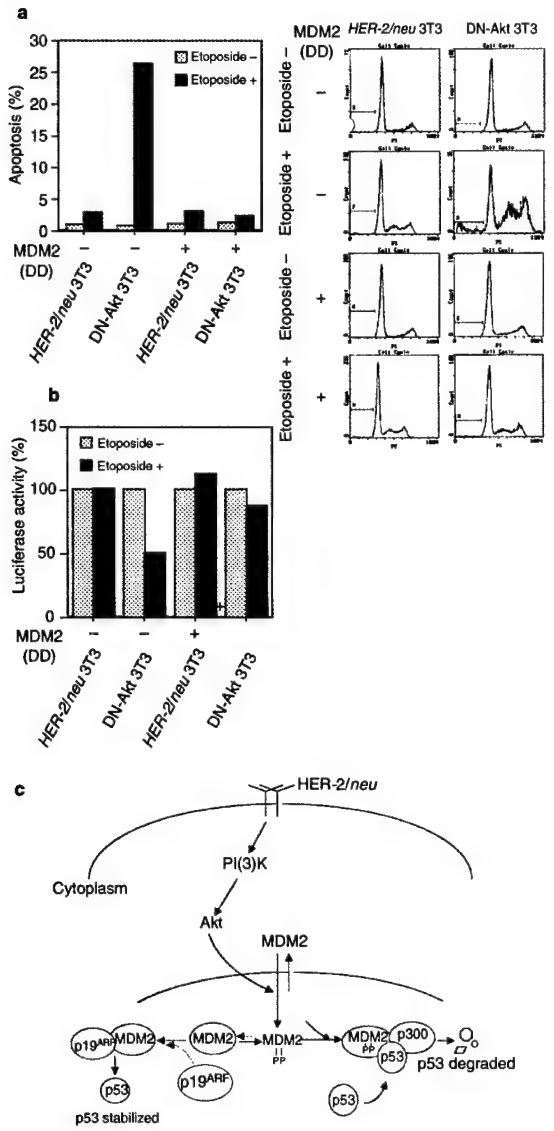

Figure 9 **Sensitization of etoposide-induced apoptosis by DN-Akt can be suppressed by nuclear MDM2 (S166,186DD). a**, Nuclear MDM2 blocks DN-Akt-mediated sensitization to etoposide-induced apoptosis. *HER-2/neu* 3T3 and DN-Akt 3T3 cells were transfected with a mimic phosphorylation form of MDM2 (S166,186DD) and then treated with etoposide (20 μM) for 48 h or left untreated. The apoptotic cells in each sample were analyaed by fluorescence-activated cell sorting as described in Fig. 1c. **b**, *HER-2/neu* 3T3 and DN-Akt 3T3 cells were transfected with MDM2 S166,186DD and a luciferase plasmid. After 12 h of transfection, cells were treated with 20 μM etoposide and incubated for another 36 h. The cell lysates were prepared and the luciferase activity in each sample was measured. **c**, A model showing how HER-2/*neu* induces the nuclear localization and altered binding of MDM2 with p300 and $p19^{ARF}$ via Akt.

uniformly in nucleoplasm in the presence or absence of $p14^{ARF}$. In the absence of MDM2, $p14^{ARF}$ was predominantly found in the nucleolus. Interestingly, MDM2 protein was not sequestered in nucleolus by $p14^{ARF}$, but rather brought the $p14^{ARF}$ out of nucleolus to the nucleoplasm. Our findings agree with recent studies that demonstrated that $p14^{ARF}$ can inhibit MDM2 function without relocalizing MDM2 to the nucleolus[35]. The cellular and subnuclear localization of MDM2 and ARF might depend on dynamic or static complex formation among MDM2, p53, ARF, Akt and some other proteins. Future systemic studies are required to clarify these issues. Taken together, our results indicate that Akt phosphorylates MDM2 at Ser166 and Ser186, and that the phosphorylated MDM2, which has a stronger binding with p300 and weaker association with $p19^{ARF}$, increases its nuclear localization and so leads to p53 degradation.

Sensitization of etoposide-induced apoptosis by DN-Akt can be suppressed by nuclear MDM2 (S166,186DD). As shown in Fig. 2, blocking the Akt pathway stabilizes p53 and therefore sensitizes cells to etoposide-induced apoptosis. MDM2 protein from the nuclear MDM2 mutant is found in the nucleus and is constitutively active without phosphorylation by Akt (Figs 5, 7). Thus, the nuclear MDM2 mutant should be able to suppress etoposide-induced apoptosis to which the cell is sensitized by blocking the Akt pathway. To address this issue, we transfected *HER-2/neu* 3T3 and DN-Akt 3T3 cells with the double mutant of MDM2 (S166,186DD) and measured their apoptosis after treatment with etoposide. As shown in Fig. 9a, the *HER-2/neu* 3T3 cells are resistant to etoposide-induced apoptosis, but blockage of the Akt pathway in DN-Akt 3T3 cells sensitizes the cells to apoptosis. However, when the S166,186DD mutant of MDM2 was transfected into DN-Akt 3T3 cells, the apoptosis induced by etoposide in DN-Akt 3T3 cells was dramatically suppressed. Similar results were obtained when we transfected with a luciferase reporter gene along with the double mutant of MDM2 and measured the luciferase activity after treatment with etoposide (Fig. 9b). Together, our results indicate that the sensitization of etoposide-induced apoptosis by the blockage of Akt pathway can be suppressed by a nuclear MDM2 mutant (S166,186DD).

Discussion

The tumour suppresser p53 plays a crucial role in controlling cell proliferation and apoptosis. In unstressed cells, p53 is latent and is maintained at low levels by targeted degradation mediated by its negative regulator, MDM2 (refs 14,17,20). The ability of MDM2 to ubiquitinate and degrade p53 depends on its E3 ligase activity and its ability to shuttle between the nucleus and the cytoplasm, which is regulated by its NLS and NES[20]. The transcriptional coactivator p300 has also been shown to form a ternary complex with MDM2 to facilitate the degradation of p53 (refs 22,23). However, the activity of MDM2 is negatively regulated by $p19^{ARF}$, which inhibits its E3 ligase activity[15]. Because the regions that control MDM2 cellular localization and binding with p300 and $p19^{ARF}$ overlap, the issues of when, where and how MDM2 is localized and interacts with functionally opposite molecules such as p300 and $p19^{ARF}$ are crucial to understanding the function of MDM2 and its regulation of p53.

In this study, we have identified a novel mechanism that controls the cellular localization of MDM2 and its interaction with p300 and $p19^{ARF}$. We show that Akt physically associates with MDM2 and phosphorylates it at Ser166 and Ser186. Our results, together with previous findings[14–29], lead us to propose a model for how Akt affects the ubiquitination and degradation of p53 (Fig. 9c). Akt phosphorylates MDM2 and induces its nuclear translocation, and the phosphorylated MDM2 binds p300 but not to $p19^{ARF}$. Binding to p300 provides a platform to facilitate the assembly of the phosphorylated MDM2–p300–p53 complex and leads to the ubiquitination and degradation of p53. Blocking the Akt pathway with DN-Akt prevents the nuclear translocation of MDM2. The unphosphorylated MDM2 in the nucleus only binds $p19^{ARF}$ but not to p300. Binding to $p19^{ARF}$ inhibits MDM2's ubiquitin E3 ligase activity and consequently stabilizes the p53 protein.

Correlation of MDM2, Akt-p and HER-2/*neu* in breast cancer

	HER-2/*neu* and Akt-p staining		
MDM2 staining	Negative (n = 12)	Positive (n = 21)	Total
Mainly nucleus	1 (3%)	16 (49%)	17 (51%)
Mainly cytoplasm	11 (33%)	5 (15%)	16 (49%)
Total	12 (36%)	21 (64%)	33 (100%)

The 33 surgical specimens of breast cancer were stained for *HER-2/neu*, phosphorylation form of Akt and MDM2 as shownin Fig. 6. The expression patterns of these three molecules in the samples from each patient was determined and summarized. Correlation of subcellular localization of MDM2 and the expression of phosphorylation of Akt was analysed by using Fisher's exact test ($P < 0.001$). A P value of less than 0.05 was set as the criterion for statistical significance.

It is evident that many of the tumours with wild-type p53 show defects in the ability to induce a p53 response. Overexpression of MDM2 is found in many types of tumour[16]. In addition, $p19^{ARF}$, the negative regulator of MDM2, is also often deleted or mutated in a wide spectrum of cancers[15]. Thus, overexpression of *MDM2* and the lack of $p19^{ARF}$ in cancers greatly increase the ability of MDM2 to degrade p53, and cause a problem for gene therapy using p53 constructs. Overexpression of *HER-2/neu* is known to activate the Akt pathway and to confer resistance to the apoptosis induced by many chemotherapeutic drugs[30]. We show that blocking the Akt pathway inhibits the nuclear translocation of MDM2 and the binding of p300, and allows the binding of $p19^{ARF}$, so sensitizing the *HER-2/neu*-overexpressing cells to DNA-damaging agents. Our results might open an avenue for developing novel anticancer therapies for *HER-2/neu*-overexpressing or Akt-activating cancers.

Note added in proof: Mayo and Donner[36] have recently reported that the PI(3)K–Akt pathway promotes the translocation of MDM2 from the cytoplasm to the nucleus. □

Methods

Materials.

The DNA-damaging agents etoposide and doxorubicin were purchased from Calbiochem. The DNA dye DAPI and anti-HA (12C5) antibody were purchased from Roche Molecular Biochemicals. The anti-p53 antibodies Ab-1 and Ab-7, and the anti-MDM2 antibodies SMP 14 and 2A10, were obtained from Oncogene Research; the anti-p300 and anti-$p19^{ARF}$ antibodies were from Santa Cruz Biotechnology. Female nude mice (*Nu/Nu*, 6 weeks old) were purchased from Harlan (Indianapolis, Indiana).

MDM2 constructs.

A *Bam*HI and an *Eco*RI site were generated near the start and termination codons, respectively, in human *MDM2* by PCR and subcloned into the expression vector pcDNA3. Site-directed mutagenesis was performed as described previously[5]. Ser166 and Ser186 in MDM2 were replaced by Asp by using the following primers: for S166D, 5′-CTAGAAGGAGAGCAATTGATGAGACAGAAGAAAATTC-3′; and for S186D, 5′-GAAAACGCCACAAAGATGATAGTATTTCCC-3′. The sequences of the wild-type and mutant *MDM2* constructs were verified by automated sequencing. To generate wild-type and mutant *MDM2* GST-tagged bacterial expression constructs, the same fragments containing the wild-type and mutant *MDM2* were subcloned into the bacterial expression vector pGEX4T-3 (Pharmacia). Expression of wild-type and mutant MDM2 proteins was induced in *Escherichia coli* strain BL21 and purified by glutathione–Sepharose chromatography (Pharmacia).

Cell culture.

NIH3T3, *HER-2/neu* 3T3, p53 and MDM2-deficient MEF, and 293T cells were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% foetal bovine serum. The DN-Akt transfectants of *HER-2/neu* 3T3 cells were grown under the same conditions except that 200 μg ml^{-1} G418 was added to the culture medium[4]. The 293T cells were transfected by the calcium phosphate technique and p53- and MDM2-deficient cells by the liposome method.

In vitro growth rate analysis.

The *in vitro* growth rates of the cell lines were assessed by the MTT assay as described previously[5].

[^{3}H]-Thymidine incorporation assay.

The proliferation rates of the cell lines were analysed by measuring [^{3}H]-thymidine incorporation as described previously[5].

In vivo tumour growth analysis.

The nude mice were randomly divided into four groups of five mice each. Two groups of mice were subcutaneously inoculated with 5×10^5 cells of *HER-2/neu* 3T3 or DN-Akt 3T3 cells per mouse. On days 5 and 7 after inoculation, one group from each cell line was giving two intravenous injections of etoposide (20 mg kg^{-1}) via the tail vein. Another two groups of mice were injected with the same volume of normal saline (the solvent for etoposide) as a control. The tumour volume (V) was measured every 2 days and calculated as $V = 0.5 \times ab^2$, where a and b are the longest and shortest diameters of the tumour, respectively. The experiment was stopped 15 days after inoculation and the results were tested statistically using two-sided log-rank analysis.

In vitro kinase assay.

293T cells (2×10^5) were transfected with 20 μg of HA-tagged CA-Akt or DN-Akt. 48 h after transfection, Akt was immunoprecipitated from cell extracts and incubated with 5 μg of purified GST–MDM2 (wild-type or mutant) in the presence of 5 μCi of [γ-^{32}P]ATP and 50 mM cold ATP in a kinase buffer for 30 min at 30 °C as described previously[5]. The reaction products were resolved by SDS-PAGE and the ^{32}P-labelled proteins were visualized by autoradiography.

Immunoprecipitation and immunoblotting.

Cells were washed twice with PBS and scraped into 500 μl of RIPA buffer. After a brief sonication, the lysate was centrifuged at 14,000 *g* for 5 min at 4 °C to remove the insoluble cell debris. Immunoprecipitation and immunoblotting were performed as described previously[4].

In situ immunofluorescent staining.

~2×10^4 cells of *HER-2/neu* 3T3 or DN-Akt 3T3 cells were plated into chamber slides for 16 h. After fixation in 4% paraformaldehyde for 1 h and permeabilization with 0.2% Triton X-100 for 30 min, the cellular localization of MDM2 was determined by using a monoclonal antibody against MDM2 (SMP14) diluted 1:1000. After extensive washing in PBS, the samples were further incubated with Texas-Red-conjugated goat anti-mouse IgG (diluted 1:500) plus Dapi (0.1 μg ml^{-1}) for 1 h. After extensive washing, the samples were examined under a fluorescent microscope (Zeiss). Nonspecific reaction of the secondary antibody was ruled out by the absence of fluorescence under the microscope.

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Ceramide does not act as a general second messenger for ultraviolet-induced apoptosis

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Ceramide has been proposed as a second messenger for stress-induced apoptosis. By characterization of murine melanoma cells and their E1A transfectants, we found several lines of evidences against the role of ceramide as a second messenger for ultraviolet (UV)-induced apoptosis. First, although E1A transfected melanoma cells were more sensitive to UV-induced apoptosis than parental cells, the relative endogenous ceramide elevation induced by UV was greater in parental cells than in E1A transfectants. Second, UV-resistant melanoma cells were more sensitive to exogenous ceramide than UV-sensitive E1A transfectants. The differential responses to UV and ceramide by E1A require the same functional CR2 domain of E1A. Third, unlike the action of UV, transient exposure (up to 2 h) of lethal dose of ceramide was not sufficient to cause apoptosis in these cells, and persistent presence of ceramide was required for processing the apoptotic process. Finally, ceramide and UV do not share a common pathway in apoptosis induction. UV-induced apoptosis was blocked by interleukin-1β-converting enzyme (ICE) inhibitor z-VAD whereas ceramide-induced apoptosis was not. Therefore, we conclude that ceramide is not a general second messenger for UV-induced apoptosis.
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Keywords: apoptosis; UV; second messenger; ceramide; E1A

Introduction

Apoptosis is initiated by a variety of physiological stimuli and environmental stresses, followed by tightly regulated cascades that activate specific proteases and nucleases that in turn carry out an ordered disassembly of cellular structures (Martin and Green, 1995; White, 1996; Wylli, 1980). Recently, attention has been directed at mechanisms by which extracellular signals activate the interleukin-1β-converting enzyme (ICE)/ced3 proteases to effect apoptosis. One important transmembrane signaling systems that may be involved is the sphingomyelin/ceramide pathway (Haimovitz-Friedman *et al.*, 1994; Hannun, 1996; Hannun and Obeid, 1995; Kolesnick *et al.*, 1994). In this proposed model, stimulation of cell surface receptors, either by cytokines or environmental stresses, activates a plasma membrane neutral sphingomyelinase (nSMase) that hydrolyzes sphingomyelin to generate ceramide and phosphocholine (Cheng and Caffrey, 1996; Devary *et al.*, 1992). Ceramide then serves as a second messenger, initiating apoptosis through the stress-activated protein kinase (SAPK/JNK) cascade (Pena *et al.*, 1997). The sphingomyelin/ceramide pathway can be activated by various stresses (X-ray, ultraviolet (UV) radiation, tumor necrosis factor alpha (TNF-α), Fas, heat shock and H_2O_2) and initiate subsequent apoptotic cascades (Kolesnick *et al.*, 1994; Martin *et al.*, 1995; Pena *et al.*, 1997; Verheij *et al.*, 1996). Thus, the sphingomyelin/ceramide signal transduction pathway appears to play an important role in mediation of stress-induced apoptosis.

The adenovirus type 5 E1A gene has been shown to sensitize cells to apoptosis induced by various stresses (Deng *et al.*, 1998; Lowe and Ruley, 1993; Mymryk *et al.*, 1994; Sabbatini *et al.*, 1995), which provides a unique tool to study the mechanism of apoptosis induction. Previously, we have shown that E1A-transfected cancer cells have increased sensitivity to apoptosis induced by various stresses and cytokines (Deng *et al.*, 1998; Shao *et al.*, 1997, 1998). Generally, E1A-expressing cells do not undergo spontaneous apoptosis; they do so only when additional stress signals are applied (Lowe and Ruley, 1993; Mymryk *et al.*, 1994; Sabbatini *et al.*, 1995; Deng *et al.*, 1998). This implies that altered stress signal transduction might play a role in initiation of apoptosis in E1A-expressing cells. Since the ceramide pathway has been shown to play an important role in stress-induced apoptosis, we asked whether the sphingomyelin/ceramide pathway is involved in E1A-mediated apoptosis in response to stresses. In this study, we characterized the possible role of ceramide in UV-induced apoptosis in both E1A transfectants and parental cells. Unexpectedly, we found that ceramide is unlikely to be a second messenger for UV-induced apoptosis.

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Results

Apoptotic process in E1A transfectants was initiated within 1 h following UV exposure

To determine the effect of E1A on cellular response to environmental stresses, we examined the susceptibility of murine melanoma cells, K1735 M2, and stable E1A transfectants of these cells (Deng *et al.*, 1998) to UV irradiation. This cell line was a metastatic variant derived from K1735 melanoma cells (Pierceall *et al.*, 1992). Both parental tumor cells (K) and vector plasmid (pSV-neo) transfectants (KSP) were resistant to UV, whereas the E1A transfectants (KA4 and KA9) were sensitive to UV-induced apoptosis (Figure 1a). The effect of E1A-mediated sensitization was dramatic: UV at a low dose (5 J/m^2) induced significant apoptosis in KA9 cells within 24 h, whereas UV at more than 100 times that dose (600 J/m^2) had only minimal effect on parental K cells (Figure 1b). For convenience of description, the results from the KA4 and KSP cells in the following studies are not shown although they behave similarly to KA9 and K cells, respectively. We next examined the kinetics of UV-induced apoptosis in E1A transfectants. The E1A-mediated apoptotic response to UV was rapid, and fragmented DNA appeared as early as 4 h after exposure (Figure 1c).

To determine the onset of biochemical process of apoptosis, we examined PARP cleavage. PARP is a DNA repair enzyme that contributes to genomic integrity and provides a critical defense against cellular stress. PARP (113 kD) is inactivated and cleaved into 85 kD fragment at the onset of apoptosis by proteases; this inactivation precedes DNA fragmentation (Bose *et al.*, 1995). UV irradiation produced no cleavage of PARP in parental cells and the PARP expression level in these cells was increased at 4 h, presumably reflecting DNA repair. However, in E1A transfectants, PARP cleavage began as early as 1 h after UV exposure and was completed by 4 h. This observation suggests that initiation of the apoptotic cascade in E1A transfectants by UV is a quick process that precedes this time point (1 h) (Chen *et al.*, 1996).

Ceramide is not involved in the E1A-mediated sensitization to UV-induced apoptosis

Because ceramide is a proposed second messenger for UV-induced apoptosis (Pena *et al.*, 1997), we asked whether the ceramide pathway is involved in E1A-mediated sensitization to UV. If so, E1A transfected cells might have either an increased ability to generate ceramide in response to UV or an increased sensitivity to ceramide. To determine whether E1A transfectants

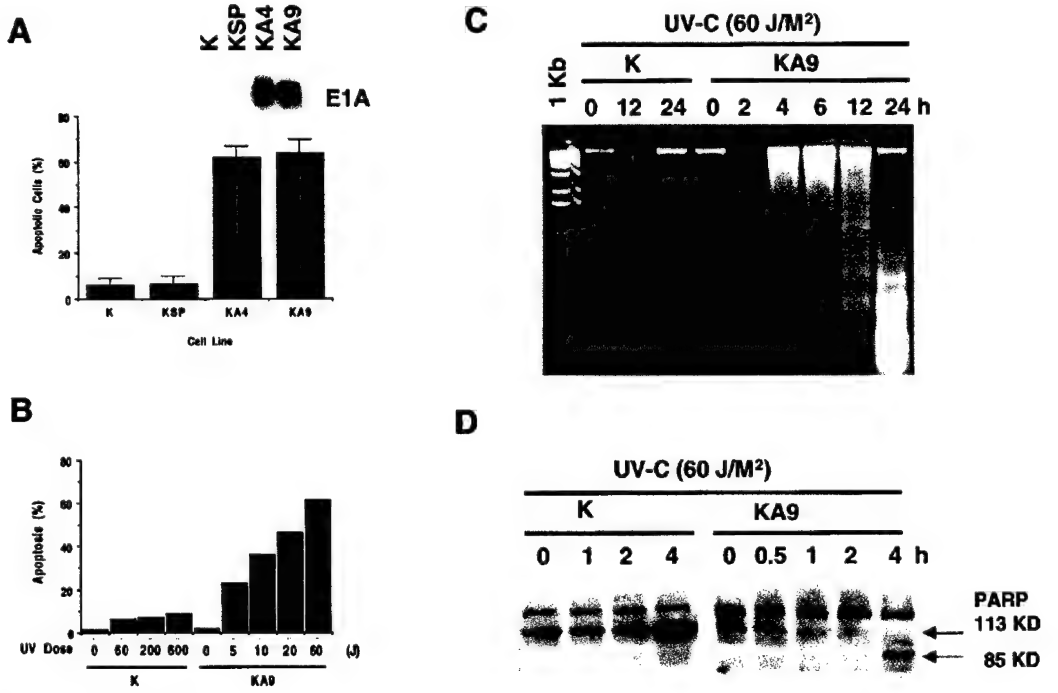

Figure 1 E1A-mediated sensitization to UV irradiation. (**a**) Induction of apoptosis (as assessed by subdiploid cell population) by UV irradiation (60 J/m^2 for 24 h) in murine melanoma cells (K), neomycin plasmid transfectants (KSP) and E1A transfectants (KA4 and KA9). The insert at the top is an immunoblot against E1A gene products. (**b**) Induction of apoptosis in K and KA9 cells by UV irradiation (24 h) at the indicated doses. (**c**) Kinetics of UV (60 J/m^2)-induced DNA fragmentation in K and KA9 cells. Similar results were obtained in KSP and KA4 cells (not shown). (**d**) Kinetics of PARP cleavage in K and KA9 cells after UV irradiation. PARP expression level was determined by immunoblot with antibody against PARP

have an increased ability to generate ceramide, we analysed the endogenous ceramide levels in both E1A transfectants and parental cells following UV irradiation. Interestingly, the magnitude of relative ceramide increase after UV irradiation was greater in UV-resistant parental cells than in UV-sensitive E1A transfectants (Figure 2a). This observation suggests that E1A-mediated sensitization does not take place at a level through an increased generation of ceramide in response to UV irradiation if ceramide functions as a second messenger. Next, we addressed whether E1A transfectants had an increased sensitivity to ceramide by examining the sensitivity of both E1A transfectants and parental cells to exogenous C2-ceramide. Unexpectedly, we found that E1A transfectants were more resistant, rather than sensitive, to ceramide than parental tumor cells (Figure 2b). Similar results were obtained by treatment of these cells with C6-ceramide (data not shown). Since E1A transfectants had neither an increased ability to generate ceramide nor an increased sensitivity to ceramide, we conclude that ceramide unlikely plays a role in E1A-mediated sensitization to UV-induced apoptosis. Moreover,

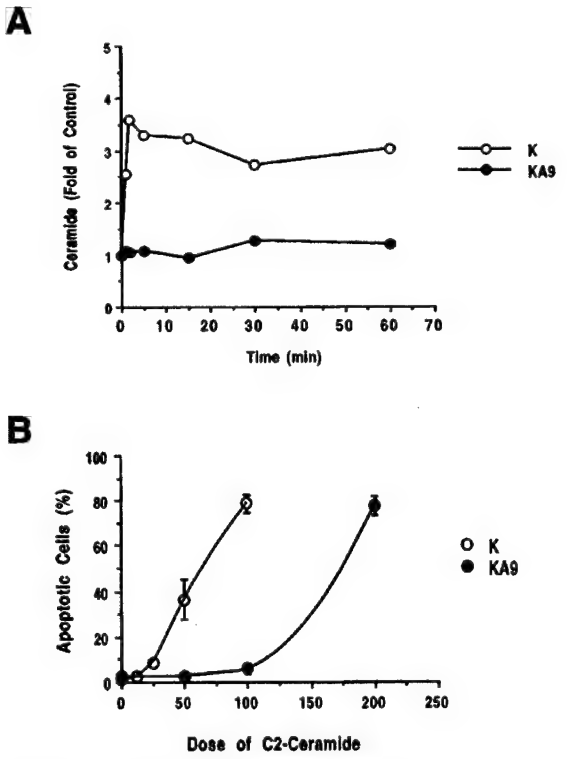

Figure 2 Comparison of ceramide generation by UV and sensitivity to ceramide in the parental murine melanoma cell line and its E1A transfectants. (**a**) Kinetics of relative endogenous ceramide levels in both E1A transfectants (KA9) and parental cells (K) were analysed at the indicated times after UV irradiation (60 J/m^2). (**b**) Induction of apoptosis in K and KA9 cells by C2-ceramide at the indicated doses, 24 h after the treatment, apoptotic cells were harvested for FACS analysis

characteristic features of UV-induced endogenous ceramide elevation and ceramide-induced apoptosis in E1A, transfectants and parental cells were inconsistent with the expected role of ceramide as a second messenger for UV-induced apoptosis.

Differential responses to UV and ceramide by E1A require the same functional CR2 domain

The E1A gene products have multiple functions on host cells by interacting with multiple cellular regulatory proteins such as p300 (Chen *et al*., 1996; Eckner *et al*., 1994) and Rb (Egan *et al*., 1989; Whyte *et al*., 1988) through different functional domains. In an initial attempt to characterize which domains of E1A responsible for sensitization and resistance to apoptosis by UV and ceramide, respectively, we examined UV- and ceramide-induced apoptosis on mutant E1A transfectants. The mutant E1A dl1101 contains a deletion at the N-terminus that is required for interaction with p300, and the mutant E1A dl1108 contains a deletion at the CR2 domain that is required for interaction with Rb (Egan *et al*., 1989; Shisler *et al*., 1996). Both DNA fragmentation and FACS analysis showed the mutant E1A dl1101 stable transfectants, K01.9, to be sensitive to UV as were the KA9 wild type E1A transfectants; but the mutant E1A dl1108 stable transfectants, K08.5, were resistant to UV irradiation (Figure 3a,b,e). These results suggest that the CR2 domain, but not the N-terminus, of E1A is required for E1A-mediated sensitization to UV irradiation. Interestingly, the sensitivity of these mutant E1A stable transfectants to C2-ceramide (Figure 3c,e) was completely opposite that of their sensitivity to UV (Figure 3b,d), suggesting that the CR2 domain is also required for E1A-mediated resistance to ceramide. Thus, differential responses to UV and ceramide by E1A required the same functional CR2 domain of E1A. This further supports the notion that ceramide unlikely plays a role in mediation of UV-induced apoptosis in E1A transfectants.

Ceramide-induced apoptosis is kinetically different from UV-induced apoptosis

It should be noticed that the ceramide pathway in parental melanoma cells is intact, since UV induced endogenous ceramide elevation (Figure 2a) and exogenous ceramide induced apoptosis in these cells (Figure 2b). However, they were highly resistant to UV-induced apoptosis (Figure 1b). This suggests that ceramide might not act as a general second messenger for UV-induced apoptosis. According to the model of the ceramide pathway, the acute ceramide elevation induced by stresses including UV, which occurs in seconds to minutes, serves as a second messenger for apoptosis induction (Hannun, 1996; Kolesnick *et al*., 1994; Pena *et al*., 1997; Verheij *et al*., 1996). If so, the apoptotic process should be inducible by a transient exposure of cells to exogenous ceramide, which mimics the kinetics of acute ceramide elevation by stresses

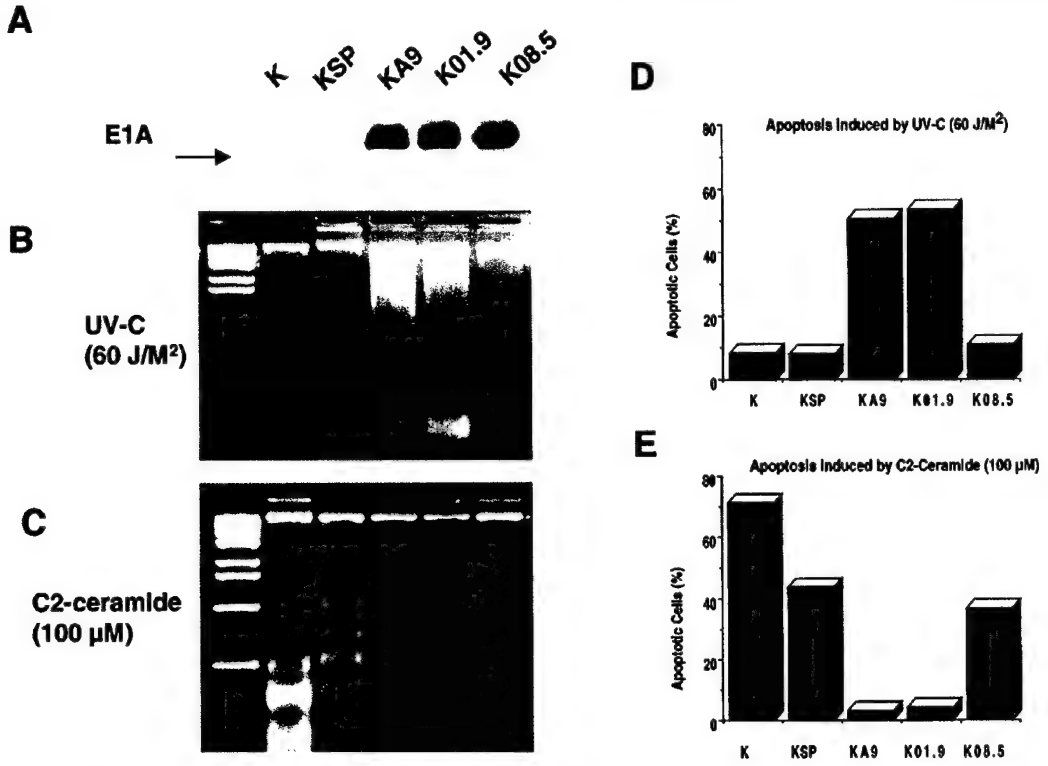

Figure 3 Different sensitivities of E1A transfectants, mutant E1A transfectants, and parental cells to ceramide and UV. (**a**) Immunoblot with antibody against the E1A gene products in different E1A and mutant E1A transfectants. K01.9 and K08.5 cells were mutant E1A dl1101 and dl1108 stable transfectants, respectively. (**b**) DNA fragmentation of various cell lines treated with UV irradiation (60 J/m^2 for 24 h). (**c**) DNA fragmentation of various cell lines treated with C2-ceramide (100 μM, 24 h). (**d**) FACS analysis of various cell lines treated with UV irradiation (60 J/m^2). (**e**) FACS analysis of various cell lines treated with C2-ceramide (100 μM for 24 h). Similar results were obtained in other clones of E1A and mutant E1A (not shown)

(Dbaibo *et al*., 1995; McConkey *et al*., 1996). Or, as the apoptotic process is irreversible, once the program is initiated, the presence of the initial signal is no longer required. We then examined the kinetics of ceramide-induced apoptosis and compared that with the effect of ceramide on cells by transient exposure. Treatment of cells with a lethal dose of ceramide (100 μM for K and 200 μM for KA9) induced a similar apoptotic kinetics in both parental cells and E1A transfectants, which began at about 2 h, and reached a plateau in about 6 to 12 h (Figure 2a). To determine whether ceramide can kinetically mimic the action of stress, both K and KA9 were transiently exposed to C2-ceramide for different periods, then removed off ceramide and replaced with normal growth medium for incubation up to 12 h. The results indicated that short exposures (up to 2 h) of cells to a lethal dose of ceramide did not cause significant apoptosis, whereas longer exposure time (>2 h) was required for induction of apoptosis (Figure 4b). Apoptotic process induced by stresses, such as UV, is known to be irreversible. Once the apoptotic program was initiated, such as 30 s UV exposure in the current study, presence of stress (UV) source is not required for continuing the process. Interestingly, by comparing the kinetic features, we found that the percentage of apoptotic cells were identical between exposure groups (cells exposed to ceramide for different times plus additional incubation without ceramide up to total 12 h, as 'expo.' in Figure 4c,d) and harvest group (cells exposed to ceramide for different time and harvested at that time, as 'harv.' in Figure 4c,d). This suggests that, additional incubation does not further increase the level of ceramide-induced apoptosis as soon as ceramide is removed. In other words, once ceramide is removed, the apoptotic process stops immediately. Taken together, the results of this analysis suggest that, (1) transient exposure of ceramide (up to 2 h) is not sufficient to induce apoptosis; (2) persistent presence of ceramide (>2–6 h) was required for processing ceramide-induced apoptosis; and (3) ceramide-mediated apoptosis stop as soon as ceramide is removed. Thus, ceramide-induced apoptosis, which requires the presence of ceramide, is kinetically different from UV-induced apoptosis that does not require the presence of stress-source. Thus, the kinetic feature of ceramide is inconsistent with the action of an expected second messenger.

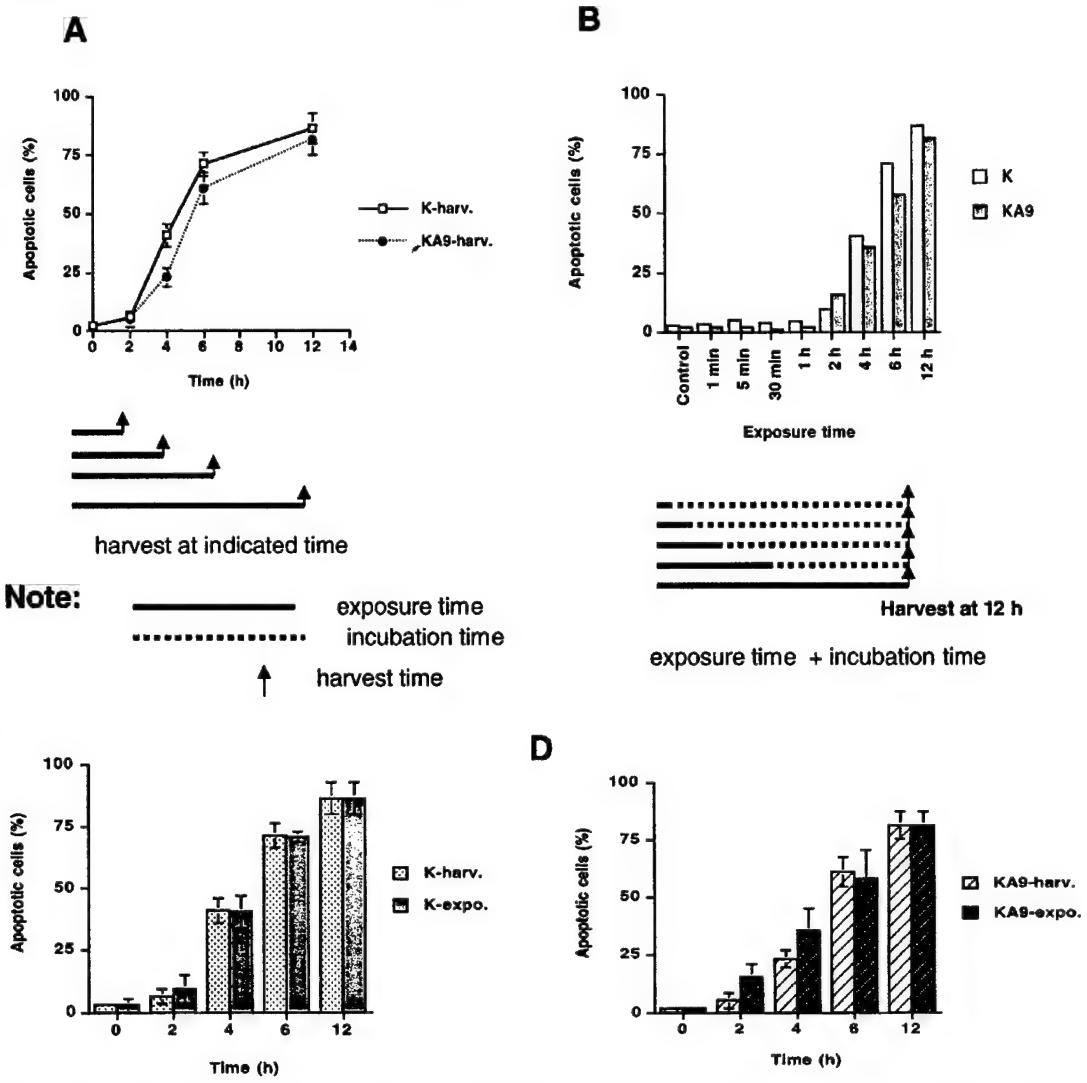

Figure 4 Kinetic analysis of ceramide-induced apoptosis. (**a**) Kinetics of ceramide-induced apoptosis. Because of different cellular sensitivities, different lethal doses of C2-ceramide were used for analysing apoptosis in K cells (100 μM C2-ceramide) and KA9 cells (200 μM C2-ceramide) (see Figure 2b). Apoptotic cells were harvested at the indicated times. (**b**) Induction of apoptosis by transient exposure of cells to C2-ceramide. Both K and KA9 cells were transiently treated with C2-ceramide (100 μM for K and 200 μM for KA9) for the indicated times. Then, the ceramide-containing medium was removed, the cells were washed once with medium and then incubated with fresh growth medium. Apoptotic cells were harvested for FACS analysis 12 h after the beginning of the treatment. (**c**) Comparison of apoptosis obtained at harvesting time ('harv.') vs exposure time ('expo.') in parental cells (K), and (**d**) in E1A transfectants (KA9). Data in **c** and **d** were obtained from the experiments in **a** and **b**. Experiments were repeated at least three times. Data are presented as mean $\pm$ s.d.

Caspases 3 is required for UV-induced but not for ceramide-induced apoptosis

Finally, we asked whether ceramide and UV share a common pathway in apoptosis induction. We assumed that UV and ceramide should share a common pathway in apoptosis induction if ceramide served as a second messenger for UV-induced apoptosis. Extracellular stress signals, including UV irradiation, have been shown to activate the ICE/ced3 proteases through signal transduction pathways to effect apoptosis (Chen *et al*., 1996). To distinguish the biochemical pathways of UV- and ceramide-induced apoptosis, we characterized the apoptotic pathways of UV or ceramide by the caspase 3 inhibitor z-VAD. Treatment of cells with z-VAD blocked UV-induced apoptosis, but did not affect ceramide-induced apoptosis in either E1A transfectants or parental cells (Figure 5a). Similar results were also obtained in the human breast cancer cell line MDA-MB-231 and 231-E1A transfectants (Figure 5b),

suggesting there is a generality of this feature. We then examined the PARP cleavage products in UV- and ceramide-treated cells by Western blot. Using Ab#1 (C2-10, Pharmigen, San Diego, CA, USA) that recognizes both full length PARP (113 kD) and its cleaved products (85 kD) (Kaufmann *et al*., 1993), we found that PARP was mostly cleaved as a 85 kD fragment in UV-treated cells, and this function can be blocked by z-VAD (Figure 5c, upper), which is consistent with UV-induced apoptosis (Figure 5a). On the contrary, only a trace amount of PARP 85 kD fragment was detected in ceramide-treated cells, which was also inhibited by z-VAD. However, this small portion of cleaved PARP 85 kD fragment that is sensitive to caspase inhibitor z-VAD can not explain the ceramide-induced apoptosis that is resistant to the z-VAD inhibitor (Figure 5a). The results suggest that caspase 3 may not be the key caspase for ceramide-induced apoptosis, and other mechanisms must be involved in the ceramide-induced apoptosis. In consistent to this notion, a novel PARP 105 kD fragment was detected in ceramide-treated cells (Figure 5c, middle). Using Ab#2 (New England BioLabs, Beverly, MA, USA) (that only recognizes cleaved PARP, but not full length PARP) on the same membrane, we detected a 105 kD fragment that was only present in ceramide- but not UV-treated cells (Figure 5c, middle). The appearance of this product was not affected by z-VAD. To compare the subtle difference in molecular weight of this product with full length and the 85 kD fragment of PARP, we then blotted the membrane with two antibodies (Ab#1 and Ab#2) together. We found that the 105 kD band is clearly different from both the 85 kD and the full length PARP (115 kD) (Figure 5c, bottom). From the protein sequence of PARP, we found that, in addition to the EVDG sequence at 215

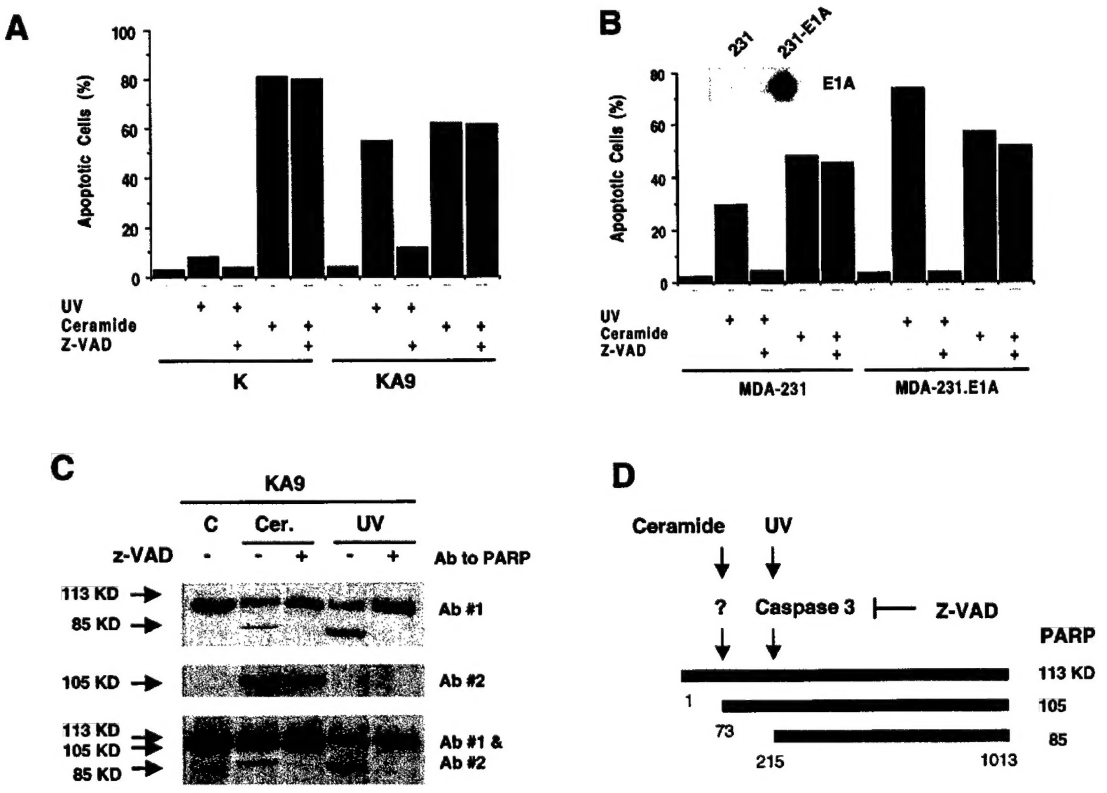

Figure 5 Ceramide- and UV-induced apoptosis go through different pathways. (**a**) The ICE inhibitor z-VAD blocked UV-induced apoptosis but not ceramide-induced apoptosis melanoma cells. K and KA9 cells were treated with UV (60 J/m^2) or ceramide (100 μM for K cells and 200 μM for KA9 cells), in the absence or presence of z-VAD (40 μM), and apoptotic cells were harvested 12 h later for FACS analysis. Similar results were also obtained for KSP and KA4 cells (not shown). (**b**) z-VAD blocked UV-induced apoptosis, but not ceramide-induced apoptosis in MAD-MB-231 human breast cancer cells, and their E1A stable transfectants. The insert at top is an immunoblot against E1A. The treatment was the same as described for **a**, except 100 μM C2-ceramide was used for both MDA-MB-231 cell line and the E1A transfectants. Similar experiments were repeated at least twice. Representative data are shown. (**c**) UV and ceramide induced different PARP cleavage. KA9 cells were treated with UV (60 J/m^2) or C2-ceramide (100 μM) in the presence or absence of 30 μM z-VAD, cells were then harvested for Western blot analysis. The membrane hybridized with the monoclonal antibody (C2-10)(Ab#1, Pharmingen) against the C-terminus of PARP (top). The membrane was stripped and re-hybridized with polyclonal antibody recognizing only the cleavage fragment of PARP (Ab#2, New England Biolabs) (middle). Then, both antibodies were combined to blot the same membrane (bottom). (**d**) The proposed model of UV- and ceramide-induced PARP cleavage. The proposed cleavage EVDG sequence is found at both 73 and 215 sites of PARP sequence

(that is cleaved by the caspase 3 between Asp and Gly), another EVDG sequence appears at the 73 site on PARP. Theoretically, cleavage of the EVDG at 73 site would result in a 105 kD product. As the combined intensity of the cleaved and uncleaved bands in ceramide-treated cells is about the same as that of the full length band in control cells (protein loading of each sample was the same) (Figure 5c, bottom), it is likely that the 105 kD band coming from cleavage at this site. Although further study is required to determine the identity of the 105 kD product and the caspase responsible for the action, the results clearly indicate that the mechanisms of UV- and ceramide-induced apoptosis are apparently different. Therefore, ceramide is unlikely to serve as a general second messenger for UV-induced apoptosis.

Discussion

The sphingomyelin/ceramide signal transduction pathway was proposed to mediate apoptosis in response to various types of stresses, including TNF-α, Fas, ionizing radiation, UV-C, X-ray, H_2O_2, and heat shock (Martin *et al.*, 1995; Verheij *et al.*, 1996). The model was mainly supported by two important lines of evidence, first that stress stimuli induce acute elevation in ceramide concentration within seconds to minutes, which precedes the cellular effects of these stimuli; and second that ectopic administration or generation of ceramide induces apoptosis, which mimics stress-induced apoptosis (Hannun, 1996; Kolesnick *et al.*, 1994; Pena *et al.*, 1997).

In this study, we examined whether the ceramide pathway play a role in UV-induced apoptosis in E1A transfectants. Unexpectedly, we found that ceramide not only does not play a role in E1A-mediated sensitization of apoptosis, but also does not serve as a general second messenger for UV-induced apoptosis. There were several evidences against the original ceramide model. First, UV-induced endogenous ceramide elevation in parental melanoma cells and E1A transfectants were inconsistent with the concept of ceramide as a second messenger (Figures 1 and 2a). Second, although E1A sensitized melanoma cells to UV-induced apoptosis, E1A also rendered these cells resistant, rather than sensitive, to ceramide-mediated apoptosis (Figures 2b and 3), suggesting that UV- and ceramide-induced apoptosis might be different. Third, the kinetics of ceramide-induced apoptosis (Figure 4) is different from that of UV-induced apoptosis, which is inconsistent with the role of an expected second messenger. Finally, caspase inhibitor z-VAD blocked the UV-mediated apoptosis and PARP cleavage, that had no effect on ceramide-induced apoptotic process. This suggests that the molecular mechanisms of UV-induced apoptosis and ceramide-induced apoptosis are different. Thus, ceramide is unlikely to serve as a second messenger for the UV-induced apoptosis.

E1A-transfected murine melanoma cells were unexpectedly found to be more resistant to exogenous ceramide-induced apoptosis than parental cells. This function, similar to the E1A-induced UV sensitivity, requires CR2 domain of E1A (Figure 3c). The CR2 domain of E1A has been shown to interact with Rb and the Rb family proteins p107 and p130 (Mymryk *et al.*, 1994; Shisler *et al.*, 1996). The Rb gene product is reported to be a downstream target for a ceramide-induced apoptosis and growth arrest (Dbaibo *et al.*, 1995; McConkey *et al.*, 1996). It remains to be seen whether interactions between E1A and Rb family proteins might play a role in the resistance and sensitization to the ceramide- and UV-induced apoptosis, respectively in the E1A-transfected melanoma cells. However, expression of E1A in MDA-MB-231 human breast cancer cells did not change the sensitivity of host cells to ceramide although E1A increased their sensitivity to UV irradiation (Figure 5b). The discrepancy between E1A effects on the murine melanoma cells and the human breast cancer cells is not clear. It may be due to different cellular context. Nevertheless, differential responses of E1A-transfected cells to UV and ceramide prompt us to re-evaluate the role of ceramide in apoptosis induction by stresses like UV. Importantly, we found that UV- and ceramide-induced apoptosis have different sensitivity to z-VAD. Moreover, the PARP cleavage patterns were different between UV- and ceramide-treated cells. Caspase 3 is known to be required for execution of apoptosis and PARP cleavage (between Asp214 and Gly215, resulting in a 85 kD fragment), which can be blocked by z-VAD. Inhibition of UV-induced apoptosis and PARP cleavage suggests that caspase 3 is required. However, ceramide-induced apoptosis failed to respond to z-VAD and different PARP cleavage was produced in ceramide-treated cells, suggesting that PARP cleavage between Asp214 and Gly215 by caspase 3 does not play a major role in ceramide-induced apoptosis, and a different apoptotic pathway is involved. Interestingly, a 105 kD band was found only in ceramide-treated cells, and appearance of this band was not affected by z-VAD. PARP was known to be cleaved by caspase 3 at the EVDG sequence between Asp214 and Gly215 amino acid residues, which results in an 85 kD product (Bose *et al.*, 1995). Another EVDG sequence was found at 73 site of PARP and cleavage at this site would result in a 105 kD fragment. Whether the 105 kD fragment is a result from cleavage at this potential site and what caspases-like activity is involved in the cleavage are interesting questions and deserve further investigation. Nevertheless, the results suggest the mechanism of UV and ceramide-induced apoptosis is apparently different.

Consistent with our conclusion, several recent observations also suggest that ceramide may not be involved in Fas- or TNF-induced apoptosis (Hus *et al.*, 1998; Karasavvas and Zaker, 1999; Sillence and Allan, 1997). However, the major evidence in these studies is that apoptosis induction was inconsistent with absolute ceramide levels. It should be noticed that the relative ceramide elevation in acute phase following stress stimuli, rather than absolute level of ceramide in later

(persistent) phase, was suggested to play a key role in stress-induced apoptosis (Hannun, 1996; Verheij *et al.*, 1996). Thus, these studies could not rule out the possibility that the apoptotic program was initiated by relative minor ceramide elevation in acute phase rather than absolute ceramide level in later (persistent) phase following stress. In the current study, we exclude this possibility by comparing the endogenous ceramide elevation by UV (Figure 2a) and sensitivity to exogenous ceramide in both parental cells and E1A transfectants (Figures 2b and 3). Furthermore, the additional kinetic (Figure 4) and biochemical (Figure 5) data provides unequivocal evidence to conclude that ceramide is unlikely to be the second messenger for UV-induced apoptosis.

Materials and methods

Reagents

C2-ceramide was obtained from Sigma (St. Louis, MO, USA). The interleukin-1β-converting enzyme (ICE) protease inhibitor z-VAD was obtained from Enzyme System Products (Livermore, CA, USA).

UV irradiation

Cells, grown to 50–80% confluence, were subjected to UV irradiation at room temperature with a source of UV-C light (254 nm). The dose of UV radiation was verified by UVX radiometer (UVP, Inc.).

Cell cultures

E1A transfectants of the murine melanoma cell line K1735 M2 and the human breast cancer cell line MDA-MB-231 were obtained by transfection with pE1A-neo which encodes both the E1A gene and neomycin-resistant gene (Deng *et al.*, 1998; Pierceall *et al.*, 1992; Shao *et al.*, 1997, 1998). Control plasmid transfectants (KSP) were obtained by transfection with backbone plasmid that encodes neomycin-resistant gene only (Deng *et al.*, 1998; Pierceall *et al.*, 1992; Shao *et al.*, 1997, 1998). The E1A mutants dl1101 and dl1108, which contain deletion mutations at the N-terminus or the CR2 domain, respectively, were obtained from Stanley Bayley (Westwick *et al.*, 1995). Mutant E1A transfectants (K01.9 for dl1101 and K08.5 for dl1108) were obtained by transfecting K1735 M2 cells with the corresponding mutant E1A plasmid plus pSV-neo, which encodes the neomycin resistance gene. Stable transfectants were selected in growth medium containing 500 μg/ml G418, and maintained in medium containing 200 μg/ml G418.

Immunoblot

Immunoblot analysis was performed as previously described (Deng *et al.*, 1998). The primary antibodies used for immunoblot were the monoclonal antibody M58 against the E1A proteins (Pharmingen, CA, USA), and the monoclonal antibody C2-10 (as Ab#1) against poly (ADP-ribose) polymerase (PARP) (Pharmingen, San Diego, CA, USA) (Kaufmann *et al.*, 1993) and the polyclonal antibody (as Ab#2) against cleaved PARP (New England Biolabs, Beverly, MA, USA). The blots were incubated with horseradish-conjugated rabbit antimouse immunoglobin (Bio-Rad Laboratories, Richmond, CA, USA) and enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, UK).

Flow cytometry, DNA fragmentation assays

Apoptotic cells were analysed by fluorescence-activated cell sorting (FACS) assay as described elsewhere (Deng *et al.*, 1998). Briefly, apoptotic cells as well as attached cells of each sample were harvested, washed once with phosphate buffered saline (PBS), and then fixed with 70% ethanol overnight at 4°C. Before analysis, cells were washed once with PBS, and then with fluorochrome solution (50 μg/ml propidium iodide (Sigma, St. Louis, MO, USA), in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma)). The percentage of subdiploid cells was considered to reflect that of apoptotic cells. Fragmented DNA was analysed by using a modified method described previously (Deng *et al.*, 1998; Nicoletti *et al.*, 1991).

Quantification of endogenous ceramide

Ceramide was quantified by the diacylglycerol kinase assay as described previously (Bose *et al.*, 1995; Dressler and Kolesnick, 1990). In brief, cells were pelleted by centrifugation and suspended in 150 μl of buffered saline solution (BSS), and then the suspended cells were extracted with 800 μl of chloroform:methanol:HCl (100:100:1 v/v/v). Lipid in the organic phase was dried in a speed vapor apparatus. Samples were dissolved in 0.1 M KOH methanol, incubated for 1 h at 37°C to remove glycerophospholipids, extracted with chloroform:methanol:1 N HCl (100:100:1) once and then dried. The dried samples were then dissolved in 60 μl of a reaction mixture containing *E. coli* diacylglyerol kinase and [γ-^{32}P]ATP, and incubated for 1 h at room temperature. The reaction was stopped by adding 100 μl of chloroform and 100 μl of methanol. The mixture was extracted once, then dried. [^{32}P]ceramide was dissolved in 5 μl thin layer chromatographic (TLC) plate using the solvent system chloroform:methanol:acetic acid (65:15:5). Incorporated γ-^{32}P was quantified by X-ray film and liquid scintillation counting (Bose *et al.*, 1995; Dressler and Kolesnick, 1990).

Acknowledgments

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